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# THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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# THE BIOLOGICAL BULLETIN

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## A QUANTITATIVE STUDY OF PHENOCOPY PRODUCTION WITH MONOCHROMATIC ULTRAVIOLET IRRADIATION

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The term phenocopy was introduced by Goldschmidt (1935) to refer to forms, produced by some experimental procedure, whose appearance duplicates or copies the phenotype of some mutant or combination of mutants. The first experiments with phenocopy production were those of Standfuss (1896) who, by treating butterfly pupae with high or low temperatures, produced adults which resembled other geographic races. Phenocopies have been produced in *Drosophila* by high temperature (Goldschmidt, 1929, 1935; Plough and Ives, 1932, 1935; Child, Blanc, and Plough, 1940), low temperature (Gottschewski, 1934), X-rays (Friesen, 1936; Waddington, 1942; Villee, 1946a), chemical agents (Rapoport, 1939), visible light (Villee and Lavin, 1946), and ultraviolet light (Geigy, 1931; Eloff, 1939; Epsteins, 1939). Geigy irradiated only very early egg stages and obtained flies with abnormal abdomens, legs, and wings. Eloff was interested primarily in the effects of ultraviolet light on crossing-over but observed some wing abnormalities when late pupae were irradiated. Epsteins irradiated larvae and pupae and produced abnormal abdomen, hemithorax, the absence of the scutellum, and abnormalities in the wings, chiefly scalloping of the distal and posterior edges of the wings. In none of these experiments was the intensity of the ultraviolet light measured.

This study was undertaken to provide quantitative data of the effects of ultraviolet light on larval and pupal stages. It was originally planned to use both 2537 Å and 2800 Å light to see if the phenocopy-producing reactions could be ascribed to changes in nucleic acid or protein metabolism. However, a light source providing 2800 Å light could not be obtained so experiments with that have been postponed. Davis (1944) made a quantitative study of the effects of ultraviolet in inhibiting the folding process in neural tube formation in chicks. By using monochromatic light of different wave-lengths obtained from a monochromator he determined the photochemical efficiency curve for the process. This was found to compare closely with the absorption curve of sterols, especially that of 7-dehydro-

<sup>1</sup> I am greatly indebted to Dr. George I. Lavin for supplying the ultraviolet lamp, filter, and meter used in these experiments and for furnishing much invaluable advice and criticism during the course of the study, and to Dr. Eric G. Ball for the facilities of his laboratory.



cholesterol, with maxima at 2576 Å and 2804 Å. Schechtman (1944) found that the inhibiting effects of ultraviolet light on the development of *Hyla* eggs were slightly stronger at 2537 Å than at other bands tested and Landen and Uber (1939) found that the inactivation of yeast by ultraviolet was greatest at 2600 Å, where 500 ergs mm.<sup>2</sup> produced 50 per cent inactivation. Stadler and Uber (1942) found that the photochemical efficiency curve of ultraviolet light in producing mutations in maize corresponded to the absorption curve of nucleic acids and Hollaender (1945) and colleagues found that ultraviolet of wave-length 2600 Å was much more efficient than other wave-lengths in producing mutations in a variety of fungi, *Neurospora*, *Trichophyton*, *Penicillium*, and *Aspergillus*.

#### MATERIALS AND METHODS

Three stocks were irradiated: a wild type and an aristopedia-Bridges (ss<sup>ab</sup>, chromosome 3, locus 58.8) stock isogenic with it (Villee, 1946b) and an independent, miniature wing (m, chromosome 1, locus 36.1) stock. Larvae were obtained by allowing large numbers of stock flies to lay eggs for a two-hour period on corn meal-molasses-agar food in half-pint bottles. The ages of the larvae are thus known to within  $\pm$  one hour. The cultures were kept at 25.5° C. before and after irradiation. At this temperature the larvae pupate within a few hours of 100 hours after they hatch from the eggs. Prepupae (white pupae) were collected frequently and the time noted. The age of the pupae at irradiation was determined from this. A total of 3,500 flies was irradiated in groups of 25 larvae or pupae.

The source of the ultraviolet radiation was a spiral quartz mercury resonance lamp, manufactured by the Hanovia Chemical Company, which emits about 80 per cent of its energy in the form of the 2537 Å line. A 120 mA. luminous tube transformer was used. The visible radiation was removed by a quartz filter cell containing a mixture of nickel sulfate and cobalt sulfate dissolved in distilled water (Backström, 1940). The lamp used had been burned well over 100 hours before the experiments began so the amount of radiation of wave-lengths shorter than 2537 Å should be negligible. The intensity of the irradiation was varied by altering the distance between the lamp and the target. The intensity was measured by a Hanovia ultraviolet meter, the target of which was fastened to a carriage on an optical bench. The larvae or pupae to be irradiated were placed in a small uncovered Petri dish on this carriage. By moving the carriage back and forth, intensity measurements were taken before and after each irradiation and always checked very closely.

The larvae to be irradiated were removed from the culture bottles, washed briefly in 70 per cent alcohol, rinsed in Ringer's solution, dried on filter paper and placed in small, uncovered, dry Petri dishes. The larvae very shortly became stuck to the glass and showed no tendency to escape from the dish. After irradiation, the larvae were moistened with Ringer's solution to free them and removed with a camel's hair brush to shell vials containing culture medium to complete development. This drying treatment had no deleterious effect on the larvae: several groups of larvae were handled in this way and dried 20 to 30 minutes without irradiation and all hatched out normally.

## RESULTS

Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. The sensitivity of flies to ultraviolet at an intensity of 44 ergs per  $\text{mm}^2$  per second for different durations of exposure, as measured by the percentages killed, is given in Figure 1.

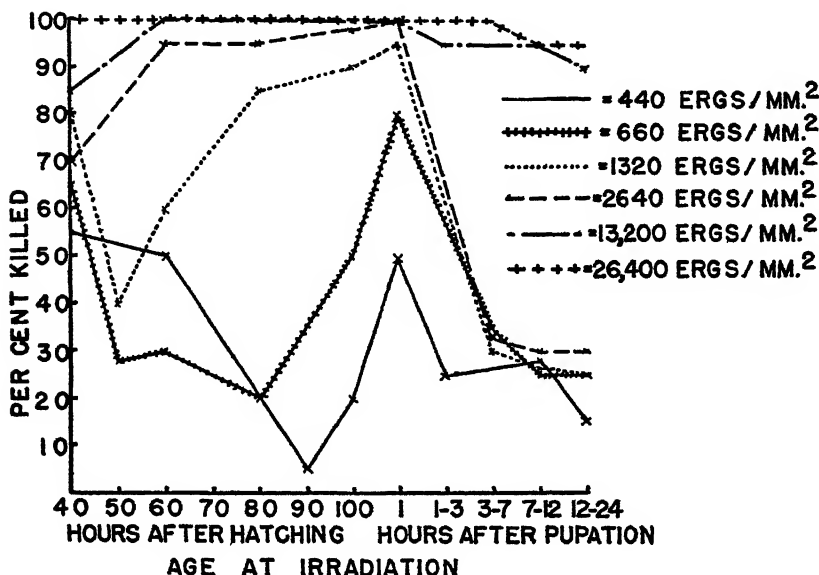


FIGURE 1. Percentage of flies killed by different total dosages of irradiation at different ages given at 44 ergs/ $\text{mm}^2$ /sec.

The percentage of flies showing phenocopies also varies with the age of the larva or pupa irradiated (Fig. 2). There seem to be two periods during the larval stage of greater phenocopy production for a given amount of radiation, one about 50 and one about 100 hours after the larvae hatch from the egg. The entire larval period from 40 to 100 hours after hatching is one of fairly high phenocopy production, higher than the first 24 hours of pupal life. Some pupae showed very high percentages of phenocopies, up to 400 per cent (400 phenocopies per 100 flies), but this was caused by the fact that the pupae can withstand more energy for the production of phenocopies without being killed. The high percentages of phenocopies were the result of irradiations with 13,200 or 26,400 ergs/ $\text{mm}^2$ . Since these dosages killed 100 per cent of the flies irradiated at most of the ages used, no lines were drawn for them on Figure 2. In 7- to 12-hour pupae, irradiations of 3,960 ergs/ $\text{mm}^2$  gave 120 phenocopies per 100 flies, 13,200 ergs/ $\text{mm}^2$ , 200 phenocopies per 100 flies, and 26,400 ergs/ $\text{mm}^2$ , 300 phenocopies per 100 flies.

The phenocopies produced included abnormal abdomen, combgap legs, abnormal thorax, small or rough eye, fused eye facets, folded, dumpy, curled or balloon wings, abnormalities in the wing veins, fused, singed or missing bristles and microchaetes,

doubled sex combs on a male prothoracic leg, and a shoulder-like protrusion growing anteriorly from the mesothorax.

The most numerous type of phenocopy produced was that involving an abnormality in the abdomen, some deformity in or the complete absence of one or more tergites. These were produced by irradiating flies in any stage of development from 40-hour larvae to 24-hour pupae. Geigy (1931) produced similar phenocopies by irradiating eggs  $\frac{1}{2}$  to  $17\frac{1}{2}$  hours old. (See Geigy for figures of the variations in phenotype produced.) Epsteins (1939) reported similar abnormali-

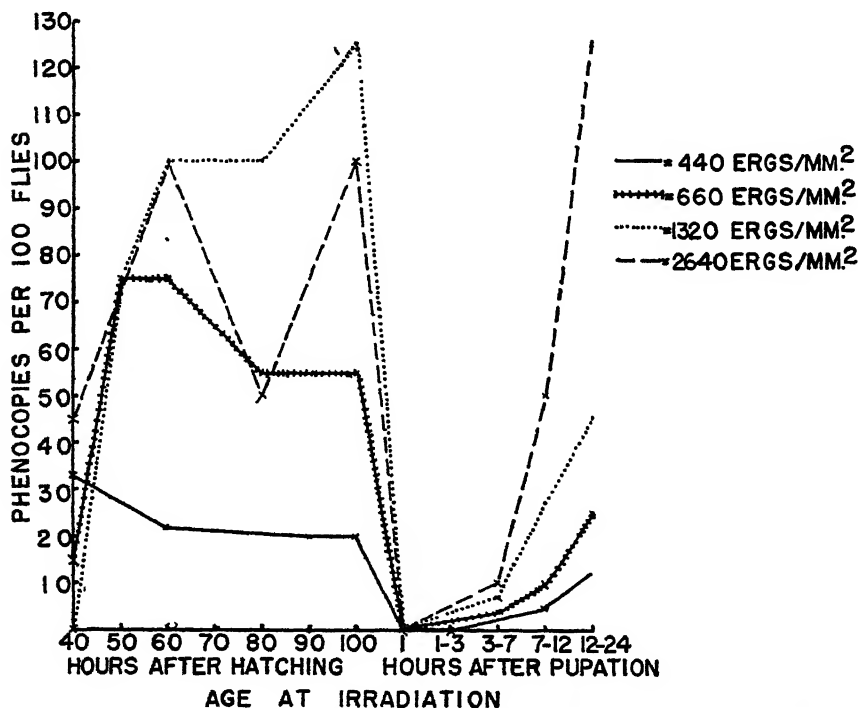


FIGURE 2. Number of phenocopies per 100 flies produced by different total dosages of irradiation at different ages given at 44 ergs/mm.<sup>2</sup>/sec.

ties produced by irradiations at times comparable to those used in this study. The large numbers of these resulting from irradiations during either the egg, larval, or early pupal stage is probably due to the fact that the dorsal abdominal imaginal discs lie close to the dorsal surface and are more readily reached by the rays than are deeper-lying discs. Eloff (1939) showed that ultraviolet light penetrates one thickness (about 10 microns) of *Drosophila* cuticle with only slight diminution of effect, but that four thicknesses screen out the effective rays.

Abnormalities in the formation of the mesothorax and scutellum occur after irradiation during the pupal period, especially between 7 to 12 and 12 to 24 hours after pupation. These include absence of the scutellum, failure of the right and left dorsal mesothoracic discs to fuse, failure of one disc to unfold properly, etc., giving phenotypes similar to those of certain grades of tetraltera (see Villee, 1942,

for figures). The eye phenocopies, small, rough, or fused eye facets, also were obtained after irradiation in the pupal period, especially between 7 and 24 hours after pupation. One fly irradiated in this period showed large, swollen legs resembling the phenotype of combgap. The wing and bristle phenocopies were produced primarily by irradiation in the pupal period, 7–24 hours after pupation. Reduction of the microchaetes occurred only after irradiation in this period. An additional period in which both bristle and wing phenocopies were produced was between 80 and 90 hours of larval life. Dumpy wings were produced only by the irradiation of the pupae.

The three stocks used in general responded similarly to irradiations of comparable developmental stages. The single exception was *aristapedia*-Bridges, which frequently developed 2- to 4-segmented tarsi when irradiated as 80- or 90-hour larvae or as 7- to 12-hour pupae. This same stock also develops very short, 1- to 4-segmented tarsi when treated with X-rays (Viltee, 1946a), low temperature (Viltee, 1943) or when combined with "growth rate" genes (Viltee, 1945), although under normal conditions it always has normal 5-segmented tarsi (see Viltee, 1946a for discussion).

Only two phenocopies were found which resembled the pervasive effects of overgrowth or abnormal histogenesis found frequently with X-radiation of 70- to 90-hour larvae (Waddington, 1942; Viltee, 1946a). One wild type male irradiated as a 3- to 7-hour pupa with a total of 5,280 ergs/mm.<sup>2</sup> developed a second pair of sex combs on the second tarsal segment of the prothoracic leg, and resembled closely a phenotype obtained with X-radiation (Viltee, 1946a, Fig. 8). One wild type female irradiated 100 hours after hatching from the egg, about 2 hours before pupation, with 2,640 ergs/mm.<sup>2</sup> developed a small palp-like outgrowth from the anterior dorso-lateral margin of the thorax, apparently identical with those obtained with X-radiation (Viltee, 1946a, Figs. 1–3).

Irradiation of larvae with ultraviolet does not cause a retardation of pupation as X-radiation does. The larvae pupate in the normal time after ultraviolet treatment; X-radiation causes a retardation of pupation of 8 to 12 days beyond the time when controls pupate (Viltee, 1946). With X-radiation there is also a marked difference in the results of irradiation of about the same dosage given at different intensities. A given dosage at high intensity causes a much higher lethality than a similar dosage at low intensity. With ultraviolet radiation, this difference is not found. A given dosage of ultraviolet light, whether given at 44, 37, 27, 17.5, or 11 ergs/mm.<sup>2</sup>/sec., produces about the same percentage of lethality and of phenocopies. It may be that the range of ultraviolet intensities used was not great enough. There is only a factor of four between the lowest and highest intensities, whereas in the X-ray experiments the high intensity was 71 times the low intensity (5,540 vs. 78 r. units per minute). However, Carlson and Hollaender (1944) found that the effects of 2537 Å light on mitosis in grasshopper neuroblasts depend simply on the total dosage and not on the intensity even when it is varied by a factor of 1500. In a later paper (Carlson and Hollaender, 1945) they found that at low total dosages (57.6 ergs/mm.<sup>2</sup>) intensities varying from 0.004 to 16.3 ergs/mm.<sup>2</sup>/sec. showed no significant differences in the effect on mitosis, but at high total doses (172.8 and 230.4 ergs/mm.<sup>2</sup>) treatments given at high intensity were slightly more effective in depressing mitosis than ones at low intensity. The dosages used in this study were higher than Carlson and Hollaender's highest but

no intensity effect was observed. Bain and Rusch (1943) reported just the opposite conditions in the production of tumors in mice by ultraviolet radiation. They found ultraviolet of wave-lengths 2800–3400 Å more effective in producing tumors when given at low intensities over long periods of time than when given at high intensities for short periods. They used much greater amounts of energy,  $116\text{--}212 \times 10^5$  ergs/mm.<sup>2</sup>, than used by Carlson and Hollaender in their experiments. The high intensity,  $1.35 \times 10^7$  ergs/mm.<sup>2</sup>/day, was about four times their low intensity,  $0.35 \times 10^5$  ergs/mm.<sup>2</sup>/day.

### DISCUSSION

The factors regulating the production of phenocopies by temperature treatments are: (1) the developmental stage at which the treatment is applied; (2) the extensity (total time) of the treatment; (3) the intensity of the treatment; and (4) the genotype of the animals treated (Goldschmidt, 1929, 1935). With ultraviolet light, the important factors are the age of the fly at irradiation, the stock used, and the total dosage of irradiation. Within the range of intensities used in these experiments, variations in intensity had no effect in changing the percentage of lethality or of phenocopies. The phenocopies produced may, in the main, be explained as due to the absorption of the light energy in individual cells or small groups of cells, probably by the nucleic acids, since 2537 Å is close to the region in which they absorb maximally, 2600 Å. Since both desoxyribose nucleic acid, located in the chromosomes, and ribose nucleic acid, located in the cytoplasm as well as in the chromosomes, absorb at the same wave-length, it is impossible to decide whether the phenocopy-producing reaction is localized in the nucleus, cytoplasm, or in both. The fact that ultraviolet of 2537 Å wave-length affects the chromosomes and retards mitosis in grasshopper neuroblasts (Carlson and Hollaender, 1944) would suggest that the phenocopy effect is also mediated by the chromosomes. Hollaender, Greenstein, and Jenrette (1941) found that 2537 Å radiation causes a depolymerization of sodium thymonucleate (desoxyribonucleate) *in vitro*. As a working hypothesis we may suppose that irradiation of *Drosophila* larvae or pupae with 2537 Å is absorbed by nucleic acids or nucleoproteins in the chromosomes of the cells of the imaginal discs near the surface. The absorption of this energy results in a physical change, a depolymerization, of the nucleic acid with a consequent upset in the structure of the gene so that it is partially or completely inactivated, with the result that development of that structure is abnormal. Since the inactivated genes are located in some body cell rather than a germ cell, a phenocopy rather than a mutation is produced. Future research may, of course, show that the phenocopy-producing mechanism is entirely different from that proposed here.

Some of the phenocopies found in these experiments suggest that the cells of the imaginal discs were killed, others suggest that certain genes were altered or inactivated, perhaps by the scheme outlined above. Only two of the phenocopies, a doubling of the sex combs on the male prothoracic leg, and a palp-like outgrowth from the anterior dorso-lateral margin of the thorax, each of which occurred only once in the course of the study, suggest the pervasive effects of abnormal histogenesis found frequently with X-radiation. These have been explained (Waddington, 1942; Villee, 1946a) by assuming that the X-rays cause the death of cells

and that the dead or necrotic cells release diffusible morphogenetic substances which result in the abnormal histogenesis or overgrowth. The same explanation may be applied to the abnormal histogeneses produced by ultraviolet radiation.

The sensitive periods for certain of the phenocopies are slightly different from those found for X-rays or temperature treatments. It is rather difficult to compare the work of different investigators, who use different stocks raised at different temperatures, but it would appear that the sensitive period for the production of dumpy wings by ultraviolet corresponds with that found by Blanc and Child (1940) for temperature treatments and that the larval sensitive period for bristle reduction by ultraviolet is identical with the temperature sensitive period determined by Child (1935). In addition there is a sensitive period for bristle reduction by ultraviolet in the pupal stage which was also found in X-radiation experiments (Waddington, 1942; Vilee, 1946a). The sensitive period for the reduction of the size of the eye by ultraviolet is in the pupal period, 7–24 hours after pupation, whereas Goldschmidt (1935) found the temperature sensitive period to be in the larval period, at an age corresponding to approximately 90 hours after hatching from the egg. Abnormalities in the thorax appear after ultraviolet irradiation in the pupal period, 7–24 hours after pupation, but in the larval period at ages corresponding to 50–100 hours after hatching from the egg following X-radiation (Vilee, 1946a). The wing phenocopies produced by ultraviolet had two sensitive periods, one in the larval period about 80–90 hours after hatching from the egg, which corresponds to the temperature sensitive period (Goldschmidt, 1935) and an additional one in the pupal period not found with temperature treatments.

The energy threshold for the production of phenocopies is slightly below the lowest total dosages, 330 and 440 ergs/mm.<sup>2</sup>, used in these experiments and varies with the age of the fly irradiated. At one hour after pupation, no phenocopies were produced by 440 ergs/mm.<sup>2</sup> but the dosage caused a 50 per cent mortality among the pupae irradiated. At other ages, from 5 to 33 per cent phenocopies (i.e., 5 to 33 phenocopies per 100 flies) were obtained with this dosage. This threshold level is of the same order of magnitude as that found by Landen and Uber (1939) for the inactivation of yeast (500 ergs/mm.<sup>2</sup> produced a 50 per cent inactivation) and by Giese (1946) for the production of abnormalities in developing echinoderms by the irradiation of sperm before fertilization. He found the threshold level for the production of abnormalities in development by irradiating the eggs of these forms to be considerably higher, on the order of 5,000–8,000 ergs/mm.<sup>2</sup> Carlson and Hollaender (1945) found a considerably lower threshold in the effects of ultraviolet on mitosis in grasshopper neuroblasts: about 100 ergs/mm.<sup>2</sup> produce a reduction of the mitotic ratio to 0.5. Davis (1944) found that the energy required to inhibit the folding process in neural tube formation varied with the wave-length of the ultraviolet used and that about 200 ergs/mm.<sup>2</sup> produced the inhibition when ultraviolet of 2537 Å was used.

It is impossible to make an exact comparison between the actions of X-rays and ultraviolet rays per energy unit, first, because their modes of action are different, and second, because only a small percentage of the X-rays are absorbed and become effective in the tissue irradiated, the rest pass through without affecting the cells, whereas ultraviolet rays are largely absorbed by tissues as thick as a *Drosophila* larva or pupa. However, it is of interest to make a rough comparison between the experiments reported here and the previous study using X-rays

(Villee, 1946a). It was found that the threshold for the production of phenocopies with X-rays was slightly below 1096 r. units. At that level from 0 to 40 per cent of the flies were killed and from 0 to 10 per cent showed phenocopies. This dosage  $D$  in roentgens may be converted to the density of absorbed energy,  $E$ , by the formula  $E = 83 D$  ergs  $\text{cm}^2$  (Cole, personal communication) to give 90,968 ergs/ $\text{cm}^2$ . The absorbed energy of the ultraviolet radiation, computed from the area and volume of the larva and the minimal threshold intensity of 440 ergs/ $\text{mm}^2$  and assuming total absorption by the organism, is 440,000 ergs/ $\text{mm}^2$ . From this it can be seen that X-radiation is approximately five times as effective per energy unit as ultraviolet radiation of wave-length 2537 Å in producing phenocopies. I want to thank Professor Kenneth S. Cole for his assistance in making these calculations.

### SUMMARY

1. *Drosophila* larvae, prepupae and pupae of various ages and genotypes were irradiated with ultraviolet of wave-length 2537 Å and in dosages varying from 330 to 79,200 ergs/ $\text{mm}^2$ . The phenocopies produced varied with the age of the irradiated fly, the stock used, and the total dosages of the irradiation. Irradiation with ultraviolet does not cause a retardation of pupation as X-radiation does.

2. Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching from the egg to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. There are two periods in the larval stage, one about 50 and one about 100 hours after hatching, of greater phenocopy production for a given amount of radiation. Irradiations during the first 24 hours of pupal life produce fewer phenocopies for a given amount of radiation than during the larval period.

3. The sensitive periods for the production of certain phenocopies by ultraviolet are compared with the sensitive periods for X-ray and temperature treatments. Some are identical, a few are different.

4. Irradiations of the same total dosage produce the same percentages of lethality and of phenocopies whether given at high or low intensities. The threshold level for the production of phenocopies varies with the age of the fly irradiated but is about 440 ergs/ $\text{mm}^2$ . A comparison is made of this threshold with the thresholds for the effect of ultraviolet on other biological systems and with the effect of X-rays on phenocopy production in *Drosophila*.

5. A hypothesis for the mechanism involved in the production of phenocopies by ultraviolet rays is discussed.

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# CYTOLOGICAL AND EXPERIMENTAL STUDIES ON THE OÖCYTES OF FRESH WATER PULMONATES<sup>1</sup>

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The organization of the ovum and the importance and relation of its inclusions such as yolk, oil, pigment, and other granules, to the ontogenic process are of considerable interest to embryologists.

Early centrifugation experiments on the eggs of many invertebrates established the facts that distribution of the visible elements apparently has no direct causal relation to the cleavage pattern of the egg or the development of the adult, and that there must be some sort of formed or underlying ground substance which could be only slightly affected, if at all, by centrifugal force (Lillie, 1906, 1909; Morgan, 1908; Morgan and Spooner, 1909; Conklin, 1910). More recently Raven and Bretschneider (1942), as the result of experiments with the eggs of the pond snail, *Lymnaea stagnalis*, have questioned the premise that distribution of visible elements in the egg has no effect on the developmental pattern. However, they have been unable to cause any atypicalities in ontogeny attributable to centrifugation and stratification of these elements.

Practically all of the centrifugation experiments directed at gaining information about the organization of the ovum or the role of the visible inclusions in development have been made on eggs already deposited, and, in many cases, already fertilized. Few, if any, have considered the egg from the point of view of its development prior to its release from the gonad, during the time when its organization might be considered in the "formative stage."

The present paper is a report of some cytological and experimental investigation on the ovarian oöcytes of two species of fresh water snails, *Lymnaea stagnalis appressa* Say and *Physa gracilis*. These species were chosen for investigation because their eggs exhibit a spiral type of cleavage which is determinate. Hence, they may be said to be "restricted" at a very early stage in development. Moreover, the potentialities of the deposited eggs of numerous species in the genera *Physa* and *Lymnaea* have already been thoroughly investigated and hence there has accumulated much information against which new research can be contrasted and properly evaluated.

The cultures of *Physa* were obtained from laboratory stocks on hand at the Biological Laboratories of Harvard University, while those of *Lymnaea* were imported to the laboratories from Wisconsin. Both cultures were kept in laboratory aquaria where they reproduced readily without special culture techniques other than those usually followed in maintaining balanced aquaria.

<sup>1</sup> The experimental work described herein was done at the Biological Laboratories, Harvard University, Cambridge, Mass.

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The body of the work may be divided into three parts. The first includes a cytological comparison of the developing oöcytes of *Lymnaea* and *Physa*. The second deals with an analysis of the effects of centrifugation upon the ovarian oöcyte, and the third is concerned with a comparison of the effects of X-radiation on the two species.

For all cytological studies, unless otherwise indicated, Champy fixing fluid was used, followed by the Champy-Kull staining procedure. The gonads were excised and then fixed, since the size of the snails made it impractical to obtain good fixation by immersion of the entire animal. Material was embedded in paraffin and sectioned at five microns.

### THE DEVELOPING OÖCYTE

As might be expected from the relative sizes of the two species, the gonad of *L. stagnalis* is considerably larger than that of *P. gracilis*. In both species, the hermaphroditic gland is found embedded in the liver on the inner side of the coiled visceral "hump." The organ is lobed and slightly more compact in its consistency than is the surrounding liver tissue. It may be found easily by tracing the prominent white and convoluted oviduct to the point where it appears to enter the body of the liver. This point marks the anterior aspect of the gonad.

The superficial histological picture of the active ovotestis presented by the two species is essentially the same. In both one finds numerous tubules in which the growth and maturation of sperms and the development of oöcytes goes on simultaneously. The oöcytes are situated along the walls of the tubules and may be identified quite early in development by their location and by their enlarged nuclei. At the earliest recognizable stage they measure about 10–15  $\mu$  in diameter, while at the time of release from the gland the diameter has increased to approximately 130–140  $\mu$ . In spite of the difference in the size of the adults of the two species, the size of the ova is about the same. For an account of the general histology of the mature ovotestis, the reader is referred to the work of Crabb (1927) in which the species *Lymnaea stagnalis* is treated at considerable length. The present report will present only sufficient descriptive background to clarify fully the position and relations of the specific regions of the gonad under consideration.

With Champy-Kull fixation and stain, the ovarian oöcytes of both *Lymnaea* and *Physa* show evidences of a clearly marked polar differentiation by the time they have reached one-third of their maximum size. The germinal vesicle is displaced slightly toward one pole of the cell and a center of accumulation of droplets or granules which are blackened by osmic acid is present in the region of the opposite pole. Yolk granules which stain red fill the cell completely and obscure details of any other cytoplasmic inclusions which might be present. The yolk granules show a slight gradation along the axis of polar differentiation, being a little finer in the region of the germinal vesicle and coarser at the opposite end of the cell (Plate 1*A* and *B*; Fig. 1*A*). A detailed study of the morphological evidences of the polarity reveals certain differences in *Physa* and *Lymnaea* which are expressed through the movements of the osmiophilic inclusions during the development of the oöcyte.

In *Lymnaea*, these black droplets appear first when the egg is about 25  $\mu$  in diameter. They arise in the vegetative region and remain there until after the

release of the oocyte. As the oocyte increases in size, the number of the globules in the mass increases until the vegetal pole becomes literally packed with them (Fig. 1B). Following shedding of the oocyte, the osmiophilic inclusions spread out from their massed apolar position and invade the region of the red-staining volky granules, with the result that the visible polarity begins to be obscured

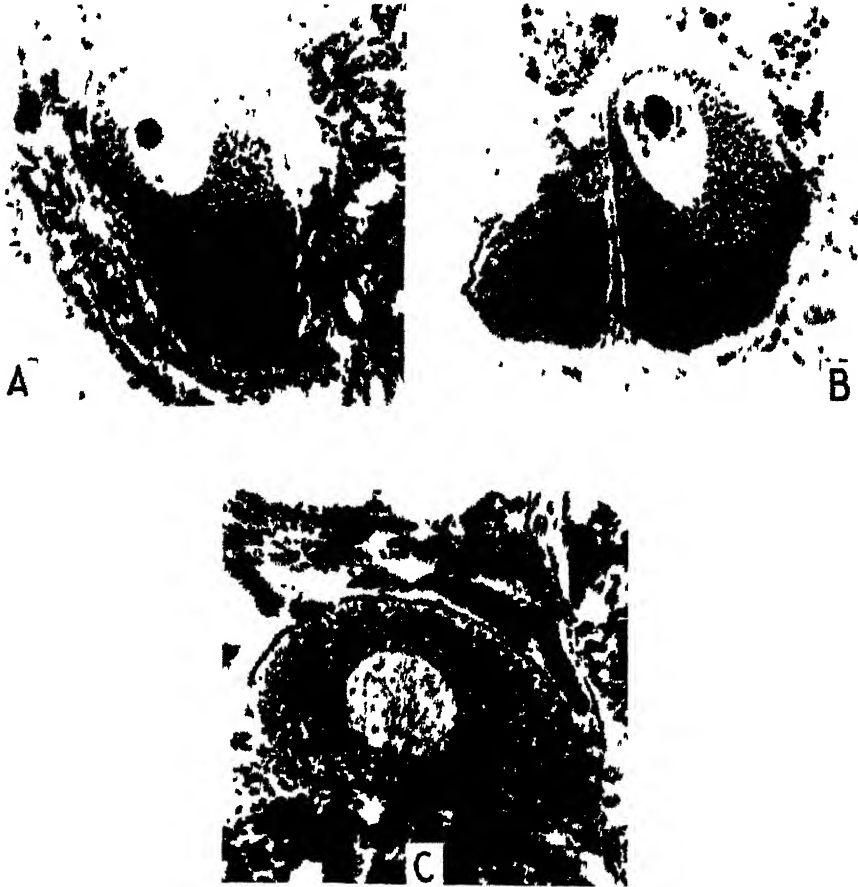


PLATE I. Photomicrographs of oocytes within their envelopes in the hermaphroditic gland  
A and B, *Lymnaca*, C, *Physa* ( $\times$  approximately 500)

(Fig. 1C). This migration starts before any signs of breakdown appear within the germinal vesicle, and progresses more rapidly in the subcortical portion of the cell. With Champy-Kull stain it is difficult to determine whether this migration is the result of factors instigated solely within the oocyte, or whether it is the result of an activation caused by fertilization. The work of Crabb (1927) on *Lymnaca* argues strongly for the latter explanation. But regardless of the cause of its origin, it is to be stressed that the pattern of migration is constant for all oocytes, and the osmiophilic material always has the same distribution in oocytes

at comparable stages of development. By the time the maturation process has begun and the nucleus is no longer intact or visible the osmiophilic mass appears equally distributed throughout the cell (Fig. 1D).

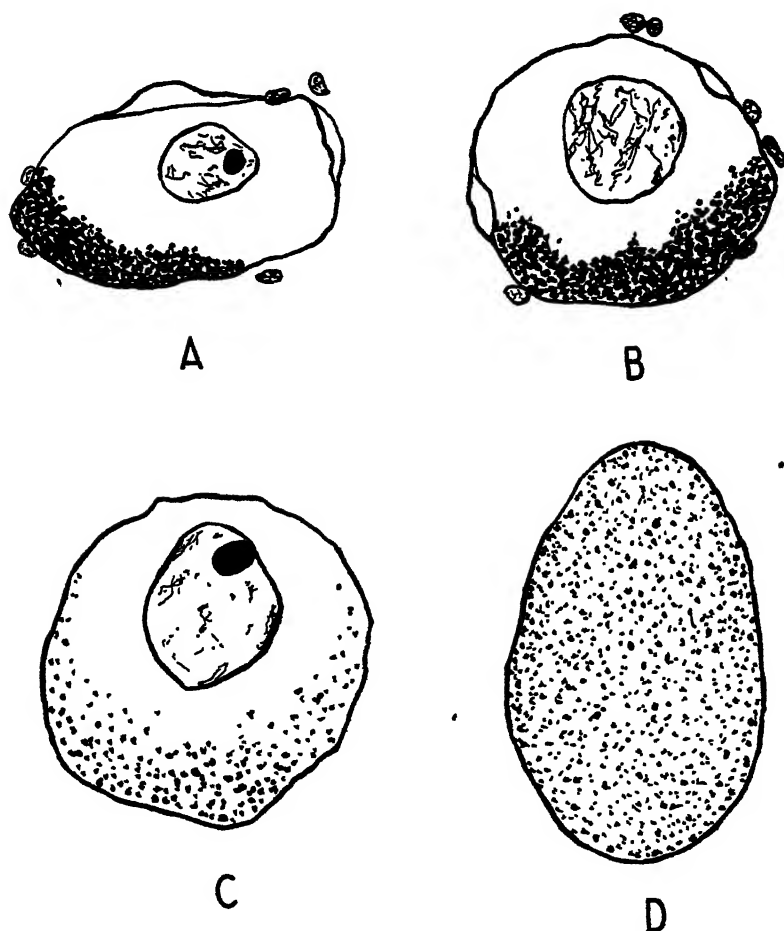


FIGURE 1. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Lymnaea*. A and B, ovarian oöcytes; C, oöcyte at the time of release from surrounding envelope; D, oöcyte in tubule of gonad just after breakdown of germinal vesicle.

In *Physa* the darkened globules appear first when the oöcyte is about 25–30  $\mu$  in diameter. Though there is some accumulation of these globules in the vegetative pole of the cell the massing is by no means as pronounced as in *Lymnaea* (Plate IC; Fig. 2A). Instead, one notes the beginning of a migration of osmiophilic material from the vegetative pole toward the animal pole even in the smaller oöcytes. As the oöcyte enlarges in its envelope the spread becomes more and more pronounced, while the massing in the vegetal pole is less pronounced. By the time the cell has attained three-fourths of its growth, the distribution of the osmio-

philic material is directly comparable with that of the released or shed oöcyte of *Lymnaea* (Fig. 2B).

Following the release of the oöcyte of *Physa* from the gland the polarity as marked by the osmiophilic globules is obscured and there is a uniform distribution of the blackened material throughout the cell (Fig. 2C). The germinal vesicle is still intact at this stage, and no signs of the beginning of the maturation process have appeared. In this respect the behavior of the dark globules differs strikingly from that observed in *Lymnaea*, where universal and even distribution is not

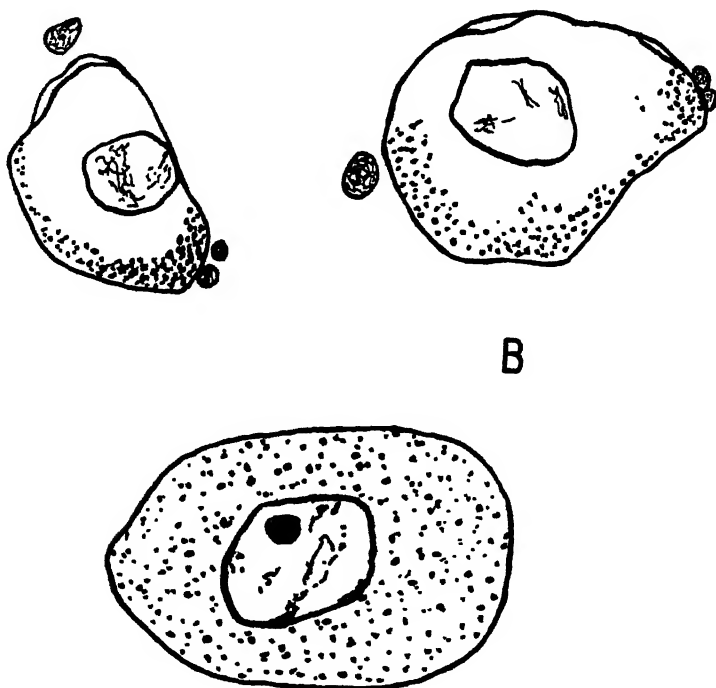


FIGURE 2. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Physa*. A, ovarian oöcyte; B, ovarian oöcyte about  $\frac{3}{8}$  developed. Compare with Figure 1C. C, oöcyte at the time of release from its envelope. Note the uniform distribution of osmiophilic material, and the fact that the germinal vesicle is still intact.

achieved until after the breakdown of the germinal vesicle. Thus the underlying system in *Lymnaea* appears more "rigid" in its composition, and though the evidence is indirect, one may speculate on the possibility that the ground substance in the oöcyte of *Physa* at the time of its release is in a more fluid physical state than that of *Lymnaea*.

No other obvious differences between species were noted, nor could other indications of the nature of the underlying cytoplasmic patterns be found. In an

effort to throw more light in this direction, living snails containing oocytes were subjected to centrifugal force so as to displace the visible inclusions of the oocytes and so further test their organization.

Single snails were placed on non-absorbent cotton in individual centrifuge tubes; the tubes were filled with water; and the snails centrifuged at approximately  $1400 \times G$  for thirty minutes. Care was always taken to select snails which were laying prolifically at the time of centrifugation. Of the thirty-six individuals of each species which were centrifuged, twelve were isolated in individual small-sized bowls and retained for subsequent study. The twenty-four remaining specimens were divided into six lots of four each, one of which was sacrificed immediately after centrifugation, and the remainder at intervals of  $\frac{1}{2}$  hour, 1 hour, 6 hours, 12 hours, and 24 hours respectively, after centrifugation. Upon excision, the gonads were fixed immediately in Champy's fluid. They were subsequently embedded in paraffin, sectioned, and stained after the Champy-Kull method. The oocytes in these preparations were then compared with each other and with those of the other lots.

#### LYMNÆA

In the sections of gonads fixed immediately after centrifugation, the contents of the oocytes are stratified into three zones; one of which is centrifugal and red (yolky), one of which is almost equatorial, relatively clear and faintly pink or



PLATE II. Photomicrographs of oocytes fixed immediately after centrifugation. *A*, *Lymnaea*; *B*, *Physa* ( $\times$  approximately 450)

colorless (cytoplasm, or hyaloplasm), and one of which is centripetal and black (oil, blackened by osmic acid, and undoubtedly the great mass of the "osmophilic material" referred to in the previous section). These three zones correspond, no doubt, to those described by Conklin (1910) in eggs of *Lymnaea* and *Physa* which were centrifuged after fertilization and deposition.

The nucleus in all cases lies in the clear or unstained zone, and, when compared with the controls, shows little indication of any displacement. The nucleoli in a great number of cells have been displaced centrifugally in the nucleus, though

this is not always the case. Not all of the yolky material within the oöcyte is massed at the centrifugal pole; some is usually found "trapped" on the centripetal side of the nucleus where it projects into the dark osmiophilic cap (Plate II*A*; Fig. 3*A*).

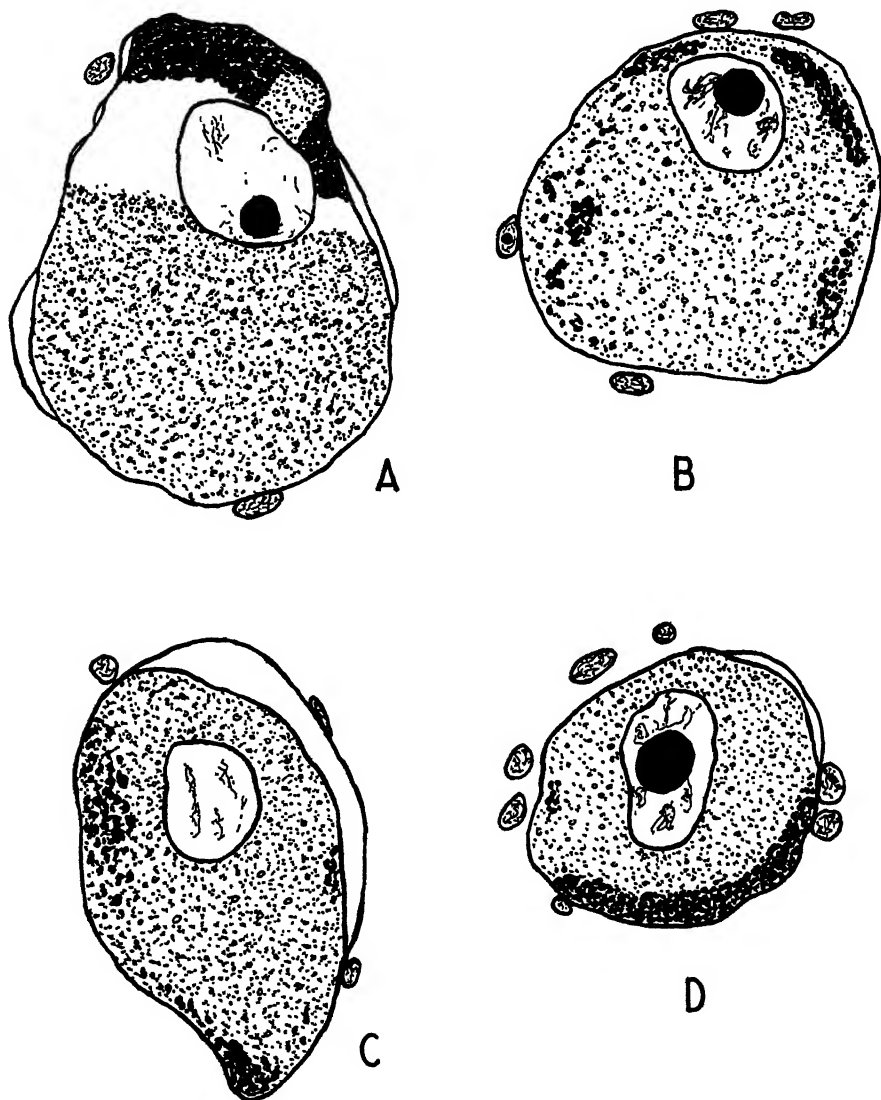


FIGURE 3. Sketches based on camera lucida tracings of oöcytes of *Lymnaea* fixed at different intervals following centrifugation. *A*, immediately after centrifugation, showing distinct layering into a black centripetal zone, clear central zone, and granular yolky zone. Granular area above germinal vesicle represents yolk "trapped" at centripetal end of cell. *B*, oöcyte fixed one-half hour following centrifugation; *C*, cell fixed one hour after centrifugation; *D*, oöcyte from preparation fixed six hours after centrifugation. Compare with Figure 1*A* and *B*.

Since the ovotestis is composed of convoluted tubules or acini, and the oöcytes are located in the walls of these acini, the orientation of the oöcytes with regard to the direction of centrifugal force varies. Stratification bears no relation to previous distribution of materials or visibly expressed polarity. This is the natural expectation based on the results of Conklin, previously mentioned.

The oöcytes in gonads fixed one-half hour after centrifugation already show a breakdown in the stratification. The "clear" hyaloplasmic area is invaded by the yolk, leaving only two of the former zones visible. The osmiophilic centripetal masses are migrating in small loose clumps from the area of former concentration, that material now being restricted to the inner cortical and subcortical regions of the cell. In the outer cortical portion between the remainder of the centripetally massed dark material and the egg surface a thin, nearly clear zone is now visible which extends over the oöcyte from the centripetal pole to a point somewhat below the middle. This zone is found one-half hour after centrifuging but is not apparent in preparations fixed immediately after centrifuging, when stratification is complete (Fig. 3B).

One hour after centrifugation the outer cortical clear zone has disappeared and the concentration of the osmiophilic substance has become diffused. Small clumps of the droplets are making their way back to their original position near the vegetative pole. The return path is not directly through cytoplasm and yolk materials but for the most part lies in the peripheral layers of the oöcyte. This phenomenon recalls the work of Lillie (1909) in which a denser "spongy" cytoplasmic core, evidently a part of the ground substance, was demonstrated in the unfertilized eggs of *Chaetopterus*. This center dense area is probably also in part responsible for the "trapping" of some yolk granules at the centripetal end of the oöcyte as described above.

At this stage, the yolk granules are uniformly distributed throughout the egg. The position of the nucleus has not changed, though those nucleoli which were displaced have left the centrifugal pole of the nucleus and have regained their more central location (Fig. 3C).

By the end of the sixth hour following centrifugation the oöcyte has, in most respects, regained its typical appearance. One or two isolated clumps of osmiophilic material may still be seen in the course of migration to the vegetative pole, but there are no other indications that the components of the cell have been displaced (Fig. 3D). By the twelfth hour all of the oöcytes have reassumed the appearance presented prior to stratification, so that it is impossible to distinguish the centrifuged ovotestis from that of the controls. The same is true for the gonads fixed after twenty-four hours.

#### PHYSA

The ovarian oöcytes of *Physa* fixed immediately after centrifugation show four zones. As in *Lymnaea*, there is a concentration of darkened osmiophilic substance at the centripetal pole, a clear band containing the nucleus, and a heavy red, yolk area occupying the greater part of the centrifugal hemisphere of the cell. In addition there may be found a fourth zone which is located at the extreme centrifugal pole. This appears as a small unstained area. In all probability it represents yolk or pigment material which does not react with the Champy-Kull stain,



and which does not separate out under the same centrifugal force in *Lymnaea*. Its position at the centrifugal pole would indicate that it is composed of the densest inclusions of the oöcyte (Plate IIB; Fig. 4A). In general there is less interference with the migration of materials by the germinal vesicle in *Physa* than in *Lymnaea*, and when "trapping" of materials does occur, the obstructed mass is always smaller than that found in *Lymnaea*.

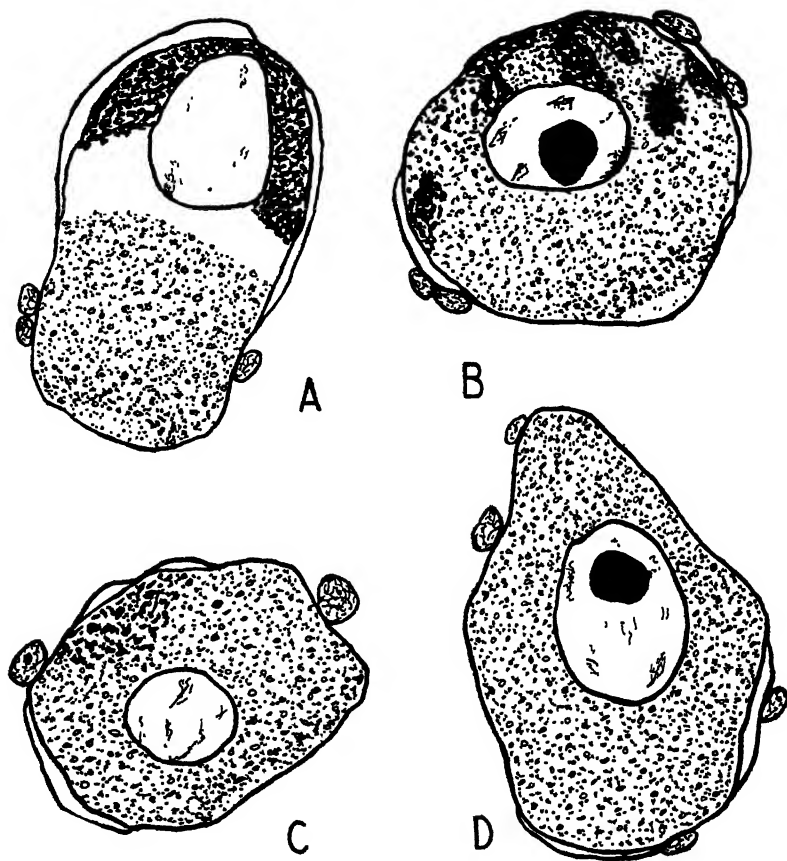


FIGURE 4. Sketches based on camera lucida tracings of oöcytes of *Physa* fixed at different intervals following centrifugation. A, immediately following centrifugation; the centripetal pole (black) oriented toward the head of the page, and the small centrifugal band (probably pigment) oriented toward the foot of the page. B, one-half hour following centrifugation; C, one hour following centrifugation; D, six hours following centrifugation. Compare with Figure 2A and B.

One-half hour after centrifugation, the materials in the oöcyte of *Physa* are fairly well redistributed. The broad, clear, hyaloplasmic band has completely disappeared; the osmiophilic concentration is rapidly breaking up into fragments which are migrating relatively freely through the cell. There is no evidence of the restricted subcortical migration noted in *Lymnaea*. The clear zone at the centrifugal pole has maintained itself, though it is, on the average, smaller (Fig. 4B).

One hour after centrifugation the dissociation of the clumped masses of blackened material is nearly complete. A small area is still to be found near what was the centripetal pole, but no other concentrations of osmiophilic material are visible. There is no return of the darkened substance to its previous location near the vegetative pole as in *Lymnaea* but instead these granules or droplets show a uniform distribution throughout the cytoplasm. The clear unstained mass at the centrifugal pole of the oöcyte has become definitely smaller and less distinct (Fig. 4C).

After six hours the oöcytes of *Physa* show no evidence of the previous stratification. The materials have been uniformly redistributed and the visible animal-vegetal differentiation which characterized the oöcytes prior to centrifugation has been eradicated. It is not regained in the oöcytes of the gonads preserved twelve and twenty-four hours after centrifugation (Fig. 4D).

The eggs of both species laid by the snails isolated after centrifuging were all collected, and their subsequent development was observed over a period of four weeks. In no case was any atypical cleavage or development found; nor did young snails on hatching show any differences in body symmetry from that observed in the normal animal.

On the basis of the above observations, it is possible to draw certain conclusions concerning the oöcytes of *Physa* and *Lymnaea*. We have seen first that the visible materials may be displaced without altering the developmental plan of the eggs. Second, the materials after being stratified, are redistributed rapidly and in the same sequence for each species. Hence we may postulate that some more basic organization or ground substance is present in the oöcyte, as in the deposited egg.

The variance in the manner of redistribution of materials in the two species strengthens the previous suggestion made on the basis of cytological observations of the normal oöcyte, that the physical state of this ground substance differs in the two species. That in *Lymnaea* definitely seems more fixed and not as fluid as that in *Physa*. The "trapping" of relatively large masses of yolk behind the nucleus, the clearly defined peripheral path of migration of the osmiophilic material, the exact reconstruction of the picture found prior to centrifugation, all point to this fact. *Physa* not only shows none of these phenomena characteristic of *Lymnaea* but under equal centrifugal force the materials of the oöcyte stratify into four zones instead of three, the densest inclusions separating out below the yolk in the centrifugal pole. Thus evidently, migration and separation is easier through the ground substance of *Physa* than of *Lymnaea*.

#### STUDIES WITH X-RAYS

The constancy of the developmental pattern resident in the the ground substance of the oöcyte was next tested by exposure of adult snails, and hence their oöcytes to X-rays.

The snails were placed in small, flat, glass containers and submitted to X-radiation at dosages of 200 r., 500 r., and 1000 r.<sup>2</sup> Fifteen individuals of each species

<sup>2</sup> In all runs reported, the X-rays were produced by a Coolidge type tube, having a water-cooled tungsten target. Dosage was continuous, and was administered at an approximately constant rate of 200 r. per minute.

were exposed at each value and were then isolated and observed at intervals. All egg capsules deposited after exposure to radiation were collected, placed in individual containers of pond water, and observed regularly throughout their subsequent development.

At 200 r. no effect of radiation was evident, either in the behavior of the adult snails or in the development of the eggs recovered. This was the case in both *Physa* and *Lymnaea*.

At 500 r. all of the individuals of *Lymnaea* exhibited a noticeable delay before any capsules of eggs were deposited. No eggs were recovered in any case prior to the fourth day after exposure. This was not true for *Physa* where capsules were recovered as early as twenty-four hours after the radiation of the adult snails.

In both *Physa* and *Lymnaea* the usual number of eggs in each capsule initiated development, though there was some increase in the number of eggs which did not complete their full development. The defects in development appeared usually after gastrulation, about the time of trochophore formation or immediately after. As in the 200 r. group, all snails which hatched were phenotypically similar to the parents.

At values of 1000 r. no egg capsules were recovered from either species.

The above results parallel, to a large extent, observations of Dr. E. V. Enzmann (unpublished). In discussions of his X-ray studies on *Planorbis trivolis*, he reported no success in efforts to cause monsters or reversals in this species. He was unable to find any aberration in early cleavage or in the final body plan of the adult snail, even though he was able to recover eggs from snails subjected to higher dosages than 1000 r.

It would therefore appear that the ground substance of the oöcyte, unless the cell is actually destroyed by X-radiation, is not affected adversely by it, at least in terms of the fundamental factors necessary to institute cleavage and development through gastrulation.

## DISCUSSION

A consideration of the results presented here in the light of reports by previous workers will readily bring out certain points worthy of special mention.

In the first place, the contention of Raven and Bretschneider (1942) that the visible elements of the egg are of far greater importance to ontogeny than had been previously thought, would seem to be strengthened. The shifting of the osmophilic materials in the oöcytes of *Physa* and *Lymnaea* not only after centrifugation but also in the normal development fits in with Raven's view that inclusions shift considerably during development, and that their presence or absence in given regions of the egg or developing embryo is of primary importance. It may be pointed out again, that the shift of the osmophilic inclusions is always the same in the oöcytes of the same species, while differing between species. This fact may well be tied in with the apparent differences in viscosity of the ground substance of the two species and is even more interesting when one considers that the cleavage pattern also differs, that of *Lymnaea* being dextrotropic, while that of *Physa* is leiotropic.

In continuing a comparison of results with those of previous workers, we find some disagreement in the section dealing with centrifugation. Conklin (1910)

and his contemporaries report that following centrifugation the eggs of fresh water snails and other invertebrates stratify into three zones; and oily centripetal, a clear hyaloplasmic, and a centrifugal yolky zone. Raven and Bretschneider, in contrast, report in *Lymnaea* a fourth zone (in their work the third zone) composed of mitochondria, or "α granules," glycogen, and other inclusions, interposed between the clear zone and the centrifugal yolky zone.

The present report is in general agreement with Conklin and disagreement with Raven and Bretschneider. True, in *Physa* a fourth zone, not reported by Conklin, was found at the extreme centrifugal pole, but this is not of especial note since in these studies the oöcytes were subjected to far greater centrifugal force than was used by Conklin. Hence some additional separation of pigment or other denser inclusions from the yolk might normally be expected. However in *Lymnaea* no trace of Raven and Bretschneider's "fourth zone" between the clear and yolky bands could be found. This may be due in part to the fact that these workers in turn used somewhat higher values of centrifugal force than did the present writer. On the other hand, Raven himself reports that in certain of his preparations fixed in Champy fluid the "α granules" cannot be made out. This may rationalize the difference, since all of the preparations used in the present study were Champy fixed. Thus it is possible that the "fourth zone" was present but did not show up.

Why Champy fixation does not demonstrate this zone is an interesting cytological question. The works of Harvey (1936, 1938) using the eggs of echinoderms and of Clement (1938) using eggs of *Physa* certainly contribute good evidence in behalf of Raven's suggestion that such a zone should be largely made up of mitochondria. But a Champy-Kull stain following Champy fixation should demonstrate mitochondria. Raven and Bretschneider can offer no explanation for the discrepancy, nor is it possible for the present writer to do so on the basis of this study.

Beyond this difference the results of the sections on centrifugation and X-radiation follow closely the findings of former workers, which demonstrated that the underlying pattern of the deposited egg cannot be altered by centrifugation or similar forces. In this respect the ovarian oöcyte is evidently no different.

#### SUMMARY AND CONCLUSIONS

The developing oöcytes of two species of fresh water snails, *Lymnaea stagnalis* and *Physa gracilis*, are studied cytologically and experimentally through the use of X-radiation and centrifugal force. As a result of the cytological studies, certain differences in the pattern of distribution of visible inclusions are pointed out. These are specific and constant within each species, but differ between species.

The constancy of pattern within the ovarian oöcytes was tested in both species by centrifuging entire snails. Displacement and stratification of the visible cellular inclusions in the oöcytes was produced, but no aberrations in subsequent development resulted. It is concluded that a fundamental ground substance is present in the oöcyte which is not affected by the stratification of visible inclusions. The manner in which the inclusions migrated from their artificially induced stratified locations was observed, and it is postulated that the ground substance of the oöcyte of *Physa* is in a more fluid physical state than that of *Lymnaea*.

Further tests on the constancy of pattern were made by exposure of snails of each species to varying dosages of X-radiation and study of the development of

eggs laid by them subsequently. It was found that as dosage increased there was a decrease in the number of eggs which were able to complete their embryonic development. No decrease was noticed, however, in the number of eggs which were able to initiate their development. It is concluded that the cytoplasmic pattern or ground substance inherent in the oöcyte is unaffected by X-radiation of dosage values below killing intensity.

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## THE RELATION OF BLOOD OXYGEN TRANSPORT TO RESISTANCE TO ANOXIA IN CHICKS AND DUCKLINGS<sup>1</sup>

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The relatively greater resistance of the new-born and young animals to complete anoxia in comparison with that of the adult animal has been studied by Himwich et al. (1941) who have advanced the hypothesis that differences in the aerobic and anaerobic energy release in the young and adult brains are the probable cause of the differences in resistance to anoxia. The newborn and young of many species of animals have been exposed to 100 per cent N<sub>2</sub>, He, and CO<sub>2</sub> and have proved to be remarkably resistant to complete anoxia in comparison to older individuals of the same species (Fazekas et al., 1941). Since there is a total lack of oxygen in the blood stream under conditions of total anoxia and since the brain is the organ most susceptible to anoxia the oxygen requirement of the brain of newborn and young animals has been studied in comparison to the energy requirement of the brain of older animals of the same species in order to find some possible basis for the greater resistance of the young and newborn to anoxia. Himwich et al. (1941) and Chesler and Himwich (1944) have demonstrated that the anaerobic energy release in the brain of the newborn is relatively greater, in proportion to the total energy requirement of the brain, than in older individuals of the same species. After birth there is a rapid increase in energy requirement as measured by the amount of respiration carried on in the brain (Tyler and von Harreveld 1942). This increase is met, chiefly, by increase in the aerobic energy release. In the newborn the total energy requirement is low and a relatively larger proportion of the requirement is met by anaerobic energy release. The survival of the newborn and young under conditions of total anoxia is adequately explained on the basis of the relatively great anaerobic energy release in the brain of the young and newborn compared to the total energy required by the brain at this stage of development to maintain the functional integrity of the organism.

Although Quastel and Wheatley (1932) demonstrated differences in the energy requirement of surviving brain slices of cat and rat brains—the rat brain had a higher energy requirement as evidenced by greater oxygen consumption of surviving brain slices—the possibility remains that in the adult animal species differences in survival at high altitude under conditions of incomplete anoxia may be explained, in part, on several bases beside differences in brain metabolism. One of these factors is differences in oxygen transport by the blood.

<sup>1</sup> This work was supported by a grant from the John and Mary R. Markle Foundation to the Pathology Department. The anti-coagulant used in these experiments was "Lequaemin" supplied by Roche-Organon Inc., Nutley, New Jersey. Some of the chicks used in these experiments were given to the University of Arkansas by the Arkansas Hatchery, Little Rock, Arkansas. Research paper 595, Journal Series, University of Arkansas.

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Rostorfer and Rigdon (1945) were able to show that malarial infection greatly reduced survival time of ducks and chicks at simulated high altitude. The effect of a severe malaria infection on the survival time was marked by the third day after the birds had been inoculated with the malarial organism. At this time there was only a small percentage of red cells infected, but a shift in acid-base balance and a reduction in the oxygen carrying capacity were developing. In the study of the effect of malaria on survival of birds at high altitude a specific difference in resistance to high altitude anoxia between ducks and chicks was reported. This difference in resistance was still evident on the third day after inoculation with malaria, although there was decreased resistance in each case.

These earlier experiments suggested to the authors that specific blood differences might be related to the specific resistance to anoxia in the case of young ducks and chicks. Accordingly, a study was made of the oxygen capacities, the oxygen dissociation curves, the amounts of hemoglobin present in blood of young and adult ducks and chickens in relation to their resistance to anoxic anoxia.

#### MATERIALS AND METHODS

White Pekin ducks and chicks of mixed breed, obtained from a commercial hatchery, between 3 and 21 days old were subjected to continuous decompression until convulsions and death occurred in a decompression chamber as previously described by Rostorfer and Rigdon (1945). Five to ten birds of the same age and species were observed during one decompression. In all experiments the rate of decompression was approximately the same with only a slight variation. The rate during the first four minutes of simulated ascent was between 1,700 and 1,800 feet per minute, between 700 and 900 feet per minute during the next 15 minutes, and 400 feet per minute during the next 40 minutes. After reaching a simulated altitude of 38,000 feet the rate of climb was such that during the next 12 minutes the altitude increased to only 40,000 feet. The altitude was recorded every 4 minutes during the decompression. These data were plotted against time and the total area under the resulting curve up to the time of death of the bird was calculated for each bird. The total area under the curve may be taken to represent the resistance of the bird to anoxia. The unit of resistance was expressed as the altitude in feet times the duration in minutes and was expressed as foot-altitude minutes.

Ducklings and chicks in groups of 7 to 12 individuals the same age were placed in the tank and decompressed until they were dead in the manner described. The ages of the various groups were 3, 6, 10, 15, and 20 days old. The average resistance to incomplete anoxia at simulated high altitude was obtained by analysis of data found by decompressing several age-groups of birds of each species. The number of birds in each separate decompression was determined by the size of the individuals.

Blood samples were obtained by heart puncture from 3 to 4 ducks or 6 to 12 chicks of the same age and pooled. Coagulation was prevented by adding 1 ml. of a 1 per cent solution of the sodium salt of heparin to each 9 ml. of blood. All red cell counts and hemoglobin and gas analyses were carried out on the pooled samples.

Oxygen capacities were determined by analysis of blood equilibrated in Barcroft tonometers with air at 38° C. Analyses were made by means of the Van Slyke manometric apparatus.

Oxygen dissociation curves were calculated for the pooled samples by equilibrating blood at a constant  $\text{CO}_2$  tension of 31 mm. Hg and at varying oxygen tensions. Three gas mixtures were used containing 3.4, 7.44, and 11.86 per cent oxygen which corresponded to oxygen tensions of  $24 \pm 0.5$ ,  $53 \pm 0.5$ , and  $83 \pm 0.5$  mm. Hg, respectively. Analyses carried out on blood equilibrated at these three oxygen tensions gave data from which the oxygen dissociation curves were calculated by the use of the formula  $y = \frac{100 Kx^n}{1 + Kx^n}$ , when the oxygen capacity of the blood was known (Hill, 1910).

Hemoglobin determinations were made on each pooled sample by the use of the Schulte and Elvehjem (1934) colorimetric method. Hemoglobin was determined, also, by calculation from the oxygen capacity. These data indicate the amount of functional hemoglobin.

Cell counts were made in the usual manner on the pooled blood and the color indices were calculated by dividing the grams of hemoglobin calculated from the oxygen capacity by the total red cell counts.

## RESULTS

In order to determine the specific resistance of young and adult ducks and chickens to the incomplete anoxia produced at simulated high altitude a series of decompression experiments were carried out in the manner described. The data represented by Figure 1 show the decreasing resistance to anoxia with increasing age. The three-day old birds of both species had the greatest resistance. There appeared to be no change in resistance between the 15- and 20-day old birds in either species. There was, however, definitely greater resistance in the duck than in the chick at all ages compared. Adult birds were not studied but there are reasons to assume that the adult duck would be more resistant to anoxia than the adult chicken.

These data indicate that the newly hatched and young birds of the same species are more resistant to incomplete anoxia produced at simulated altitude. This corresponds to the resistance of young and newborn mammals studied by Himwich and others. The data indicate, also, a specific difference in resistance to incomplete anoxia between ducks and chicks. Ducks 20 days old decompressed in the usual manner succumbed at altitudes between 34,000 and 36,000 feet, while chicks 20 days old were all dead at 27,000 feet when the decompression was at the same rate as that for the ducks.

There is little doubt that specific differences in oxygen capacity and color index exist among animals. This difference might possibly bear some relation to resistance to anoxia. The oxygen capacities and color indices were determined for all blood samples. Determination of oxygen capacities of duck and chick blood revealed that the duck blood had considerably greater oxygen capacity. Duck blood from 20-day old birds had an average oxygen capacity of 12.27 volumes per cent. Chick blood from birds the same age averaged only 10.47 volumes per cent.

The color indices of the 20-day old duck varied around  $3.6 \times 10^{-11}$ . Rostorfer and Rigdon (1946) found that the color indices gradually increased with age of the young ducks. The color index of chicken blood is approximately  $3.1 \times 10^{-11}$  and there seemed to be no increase in the color index with age as in the duck. The



color index of blood taken from the adult chicken was  $3.3 \times 10^{-11}$ . There was an appreciable difference in total red cell counts between the duck blood and the chick blood. The average count for the pooled blood of the chick was 2.2 million and the average count of the pooled blood of the duck was 2.6 million per cubic millimeter.

The chick blood in comparison to the duck blood had fewer red cells, decreased color index, and lower oxygen capacity.

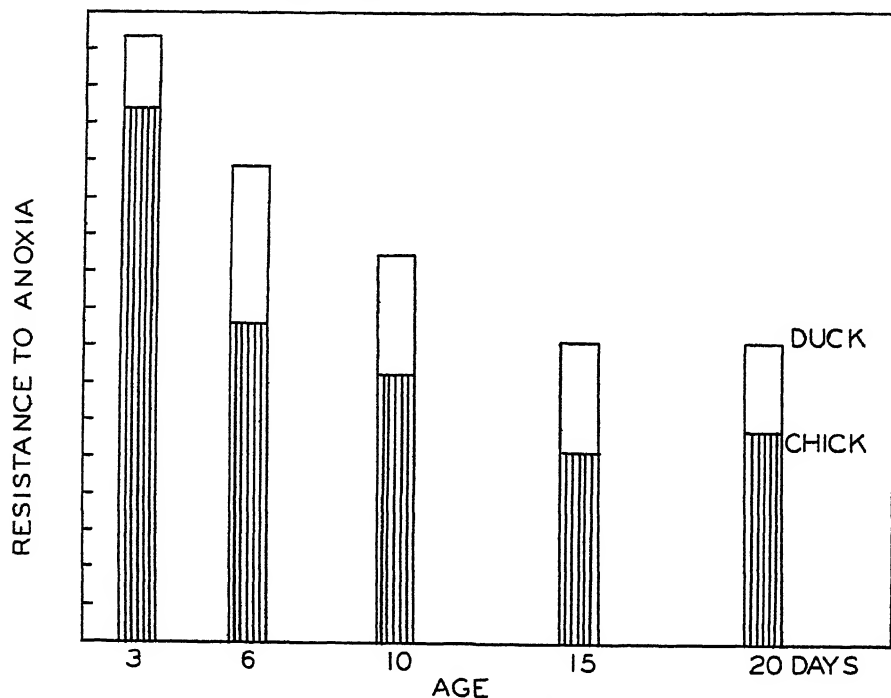


FIGURE 1 is a graphic representation of the data on the study of relative resistance of chicks and ducks to simulated altitude. Resistance is expressed in feet of altitude times the duration at altitude in minutes. Each age group represents the average data of 10 to 20 ducks or chicks.

A considerable difference between the amount of hemoglobin determined colorimetrically and that calculated from the oxygen capacity was found for young and adult ducks by Rostorfer and Rigdon (1946). A similar study was carried out on young and adult chicks. The comparison is made in Figure 2 which represents the relationship between the amount of hemoglobin determined colorimetrically and the amount of hemoglobin calculated from the oxygen capacity. The linear expression was obtained by determining the amount of hemoglobin colorimetrically and by calculation from the oxygen capacity of a diluted, a normal, and a concentrated sample of the same pooled blood. Cell counts of the three samples, dilute, normal, and concentrated, were made and the color index of each determined. There was usually close agreement between the color indices of the three samples. The young chicks and ducks carry less oxygen per gram of hemoglobin than the

adults based on the colorimetric hemoglobin. There was a slower shift toward the adult relationship with increasing age in the duck than in the chick. Thirty-day old chicks were found to have hemoglobin which corresponded to that found in the hen. The linear relation between oxygen capacity and the acid hematin of the adult chicken blood was the same as that of the young duck.

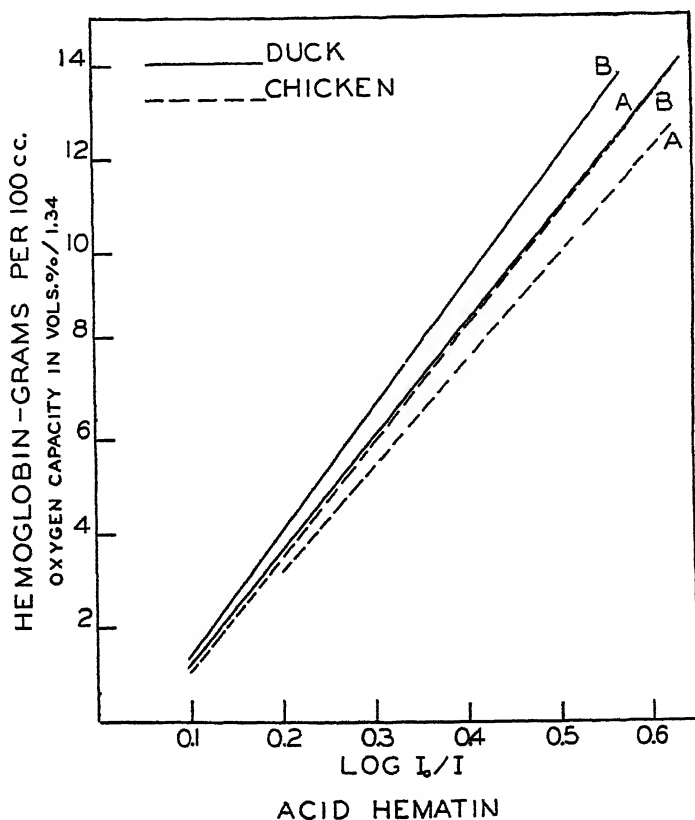


FIGURE 2 is a graphic representation of the relationship of the amounts of hemoglobin calculated from the oxygen capacity in comparison to the amount of hemoglobin determined by photo-electric colorimetry in the blood of young (A) and adult (B) domestic ducks and chickens.

The equilibrium between hemoglobin of bird blood and oxygen at various oxygen tensions has been investigated by Wastl and Leiner (1931) whose work indicated that blood of the duck and of the pigeon does not have typical oxygen dissociation curves. Redfield (1933) points out that such curves for blood containing hemoglobin had not been described previously but were characteristic of the blood of invertebrates. Christensen and Dill (1935) were not able to confirm the work of Wastl and Leiner, but found that the blood of ducks and other birds developed equilibria between hemoglobin and oxygen in the classical manner except at very low oxygen tensions. This discrepancy they attributed to a systematic error of equilibration. Rostorfer and Rigdon (1946) found considerable difference be-

tween the dissociation curves for young and adult ducks. The blood of the adult birds gave a typical sigmoid curve while that of the young ducklings had a greater oxygen uptake at lower oxygen tensions than would be indicated by calculation from points of equilibrium at higher oxygen tensions. However, the deviation was slight.

Bloods of young and adult chickens were investigated in similar manner. Figure 3 compares the oxygen dissociation curves of adult ducks and chickens. There was a considerable difference between the oxygen uptake of the blood of the

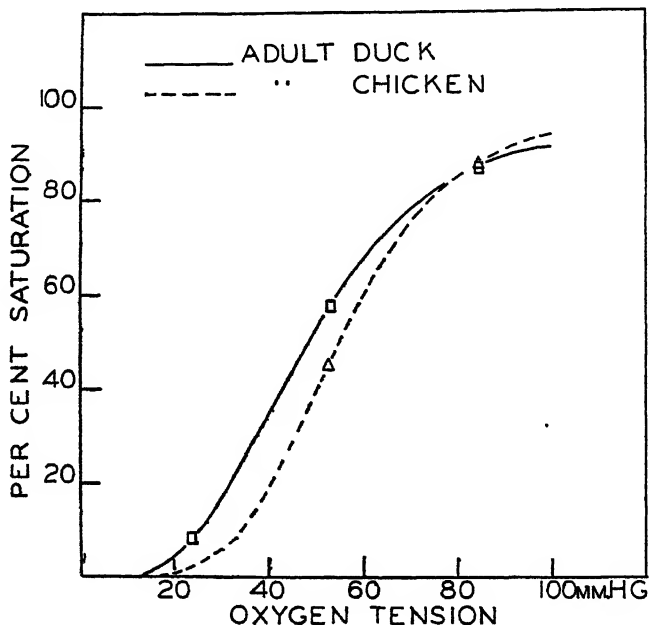


FIGURE 3 compares the oxygen dissociation curves for the adult duck and chicken at a  $\text{CO}_2$  tension of 31 mm. Hg.

adult duck and the adult chicken at various oxygen tensions. The adult duck blood had about the same percentage of saturation at ordinary oxygen tensions but was capable of carrying a larger amount of oxygen than chick blood at low oxygen tensions.

Figure 4 compares the dissociation curves of the blood of the adult and of the young chicken. Although the curve for the blood of the young chick represents the data obtained from one sample of pooled blood only, it is reasonable to assume that it is representative. In general, the blood of the young chick appeared to have a greater percentage of saturation at all levels of oxygen tension than the blood of the adult chicken. In contrast to the blood of the adult duck, the blood of the young chick had a higher percentage of oxygen saturation at higher oxygen tensions but a slightly lower per cent of saturation at low oxygen tensions.

In general, the blood of the duck in comparison to chicken blood had greater oxygen capacity, contained more functional hemoglobin, and showed less dis-

crepancy between the colorimetric hemoglobin and the hemoglobin calculated to be present from the oxygen capacity. Duck blood had a larger number of cells and a higher color index than chicken blood. The chemical behavior of duck blood in comparison to chicken blood, as regards the oxygen dissociation curves, indicated that the duck blood would be more efficient in the transport of oxygen under conditions of incomplete anoxia. The blood of the duck in comparison to that of the chick would appear to be more capable of maintaining the brain and other vital organs in a viable condition for a longer time under conditions of simulated high altitude and resulting anoxia.

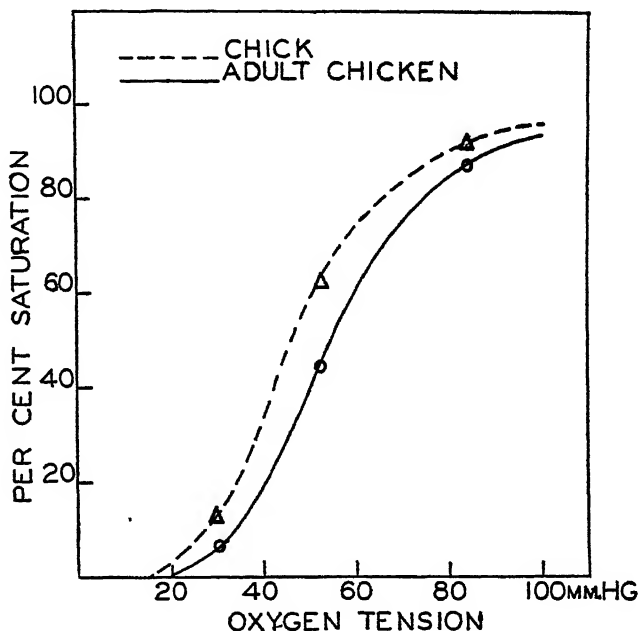


FIGURE 4 compares the oxygen dissociation curves of young and adult chicken blood at a  $\text{CO}_2$  tension of 31 mm. Hg.

It is not unreasonable to assume that some of the difference in specific resistance to anoxia of the duck and the chick is caused by the greater efficiency of the blood of the duck at low oxygen tensions. However, a study of the anaerobic and aerobic energy release compared to the total energy requirement of the brain of the two species in question has not been made as yet. One might well assume that there are specific differences in brain metabolism.

#### SUMMARY

A study of the resistance of ducks and chicks to incomplete anoxia produced at simulated altitude is reported. The newly-hatched and young birds had greater resistance than older birds. The duck was found to be more resistant than the chick to incomplete anoxia at all ages studied.

A study of the oxygen carrying capacities, total red cell counts, color indices, the amount of hemoglobin, and the oxygen dissociation curves of the blood of the duck and the chick is reported.

The blood of the duck has greater oxygen capacity, higher total red cell count, higher color index, more hemoglobin and a higher percentage of saturation of the blood with oxygen at low oxygen tension when compared with blood of the chicken.

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# THE FOOD-VACUOLE IN *PARAMECIUM*<sup>1</sup>

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## INTRODUCTION

The food-vacuole has been more extensively studied in *Paramecium* than in any other organism. There is, however, still much diversity of opinion concerning the processes involved in its formation and movement and in the changes that occur in it. These phenomena will be considered in the following pages.

## MATERIAL AND METHODS

Four species of *Paramecium*<sup>2</sup> were used in the following observations, namely, *caudatum*, *multimicronucleatum*,<sup>3</sup> *aurelia*, and *trichium*. All the specimens used were obtained from pure cultures maintained in the laboratory.

<sup>1</sup> I am indebted to Dr. W. J. Bowen for very efficient assistance in some of this work, and to the artist John S. Spurbeck, for expert service concerning the figures.

<sup>2</sup> Dr. D. H. Wenrich very generously identified the species used.

<sup>3</sup> The name "*multimicronucleatum*" is so long and unwieldy that the abbreviation, *nucleatum*, will hereafter be used in place of it.

Paramecia are usually so active that it is very difficult to study them under high magnification. Various methods have been used to quiet them, e.g., addition of narcotics (Bills, 1922), addition of yeast-cells stained with congo red (Buck, 1943), increase in viscosity (Marsland, 1943; Moment, 1944; and others). All these methods and also reduction in temperature were tried. The best results were obtained with the second method and some modifications of it.

It was found that locomotion can readily be stopped without apparent injury by means of narcotics or by increasing the viscosity of the culture fluid, but that when locomotion ceases under these conditions, feeding does also. It was also found that while movement decreases greatly with decrease in temperature, it does not decrease sufficiently to make observation under high magnification practicable until after the paramecia have been actually frozen and killed.

Nearly all the observations were made on preparations made as follows: A drop of solution containing numerous paramecia from a young, vigorous culture was mounted between two small ridges of vaseline on each of several slides. Yeast-cells stained with congo red (Buck, 1943) were added to some, chinese ink or carmine to others, and an abundance of zooglea and a little neutral red or Nile blue to still others. Then all were covered with cover-glasses and sufficient fluid removed from some to flatten the paramecia considerably. All the preparations were kept in a damp chamber when not under observation.

The paramecia in these preparations usually lived for several days. They were very active at first but they soon became quiet, although it sometimes required 24 hours or more, and in some of the preparations they became so quiet that given individuals could be studied continuously for several minutes, even under an oil-immersion objective. Nearly all the quiet paramecia, even those which had been flattened, fed vigorously. In some of these, especially those which were flattened, the structure of the feeding apparatus, the activity of the cilia and the movement of particles in it, and the formation of the food-vacuoles could be clearly seen.

It was found that the paramecia from young, rapidly growing cultures become quiet much more readily than those from old declining cultures. This is doubtless, at least in part, due to difference in the hydrogen-ion concentration of the cultures. At any rate, it was observed that if a trace of alkali is added to fluid from young cultures the activity of the paramecia in it increases greatly and that if a trace of acid is added to fluid from old cultures the activity of the paramecia in it decreases.

#### THE FEEDING APPARATUS

Numerous observations were made on the feeding apparatus in the four species of *Paramecium* listed above. The following conclusions were reached: The feeding apparatus is essentially the same in structure in the four species studied. It consists of a shallow ciliated groove (the oral groove) which extends from the anterior end to slightly beyond the middle of the body, a ciliated depression (the vestibulum) at the posterior end of the groove, a ciliated tube which extends from an opening in the floor of the depression (the mouth) backward into the body, and some fibers which extend from the distal end of the tube toward the posterior end of the body.

The tube is called pharynx by some, cytopharynx, gullet or esophagus by others, and a portion of it pharynx and the rest esophagus by still others. I shall call it

pharynx. The pharynx can be seen distinctly in living specimens. It extends from the mouth directly toward the center of the body a short distance, then turns backward sharply and proceeds parallel with the surface of the body for some distance, then turns sharply again but nearly at right angles to the first turn and ends almost immediately in an elliptical opening which leads into the forming food-vacuole which I shall designate "esophageal sac" (Fig. 1).

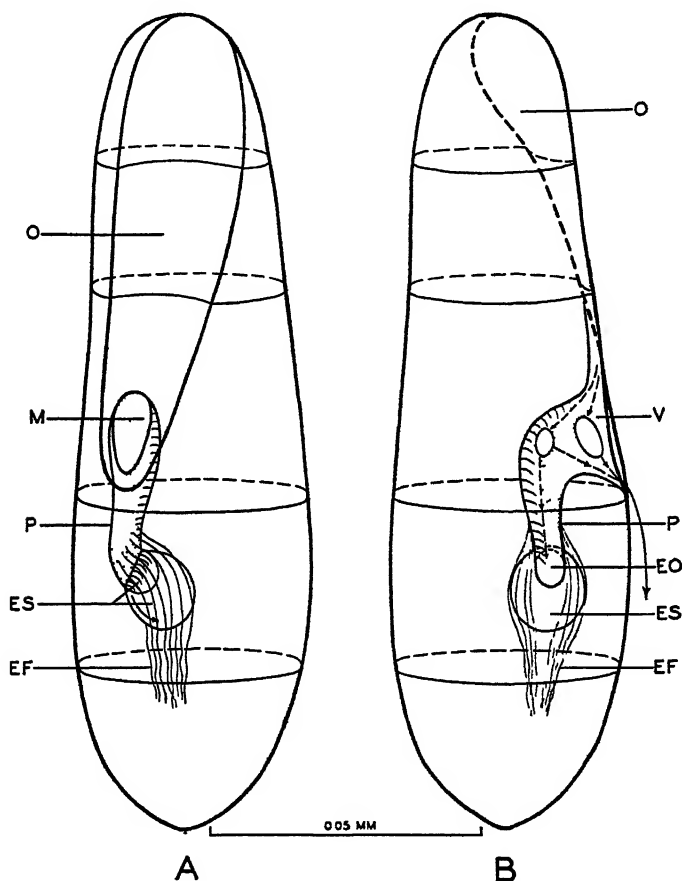


FIGURE 1. Camera outline of *Paramecium aurelia* showing the feeding apparatus and its relation to the rest of the body. A, oral groove and mouth facing upward; B, oral groove and mouth facing to the right; O, oral groove; M, mouth; V, vestibulum; EO, esophageal opening; P, pharynx; ES, esophageal sac; EF, esophageal fibers.

Nirenstein (1905) and others maintain that the mouth is a fixed oval opening. But Frisch (1937) says it "is a narrow slit bounded by a raised, thickened border in the form of an elongated oval, somewhat like lips" and that it is sometimes closed. His observations were made on an exconjugant individual, beginning immediately after it had separated from its mate and continuing for one and one-half hours. I have seen the mouth under the oil-immersion objective in many individuals under



various conditions but not in any immediately after conjugation. In all these, the mouth was continuously distinctly oval in outline, and I did not see any indication of change in form or size. It may well be that the phenomena observed by Frisch occur only for a few hours after conjugation.

If a paramecium is so oriented under the cover-glass that the anterior end is directed from the observer and the oral groove is to the left and faces upward, one can see down through the mouth into the proximal end of the pharynx and one can see the esophageal sac extending at right angles from the opening at the distal end. If the paramecium now rotates on its longitudinal axis  $90^\circ$  to the left, one can see the sharp curve in the pharynx at the proximal end and one can see down through the esophageal sac into the distal end of the pharynx. A more distinct view of this sac (the forming food-vacuole) is, however, obtained when the oral groove is to the right and faces downward, for the distal end of the pharynx is now nearer the upper surface of the body (Fig. 1).

The esophageal sac consists of a thin elastic membrane which separates the content of the sac from the cytoplasm. It changes greatly in form and size as the food-vacuole develops.

The inner surface of the pharynx contains numerous cilia. This can be seen clearly in living specimens, but no details can be made out concerning their arrangement or their size or their action, except at the distal end where a number of long cilia can be seen to beat vigorously into the forming food-vacuole.

Gelei (1934) found, in observations on fixed and stained paramecia, that there are in the pharynx two bands of cilia and that one of these bands, the "penniculus", extends from the anterior left edge of the pharynx nearly to the posterior oral edge and the other (called "Vierermembran") from the anterior aboral edge to the posterior oral edge. He maintains that the former usually contains eight rows of short cilia and the latter four rows of long cilia which at the distal end of the pharynx extend into the forming food-vacuole. Lund (1933, 1941) agrees with Gelei in reference to the composition, the location and the extent of the former and the composition and the location but not the extent of the latter. He holds that the latter does not extend to the distal end of the pharynx but that there is at this end a "pouch" which contains a "heavy tuft of cilia."

The results of my observations are in full accord with Gelei's contention. I could not see anything that resembled a pouch at the end of the pharynx, but I could see distinctly long cilia at this end, which extended into the forming food-vacuole. These however, clearly appeared to be at the end of the "Vierermembran" not in a partially separated pouch (Fig. 2).

Bozler (1924) maintains that there are attached to the right wall of the pharynx about half-way up, some ten long fibers which extend nearly to the posterior end of the body, and that these fibers are fixed in position, fairly rigid and in the same plane. He calls them "Schlundfaden" and says similar fibers occur in other protozoa and that Schuberg (1890) first discovered them in observations on *Stentor*.

Gelei (1934) and Lund (1933, 1941) confirm Bozler in reference to the presence of long fibers attached to the pharynx in *Paramecium* but they do not agree with him in the contention that the fibers are fixed and in one plane. Gelei holds that they are attached to the "right wall" of the pharynx but near the proximal end and that they are not all in one plane. Lund maintains that they are attached near the distal end of the pharynx on all sides and are not fixed. He says (1933, p. 55):

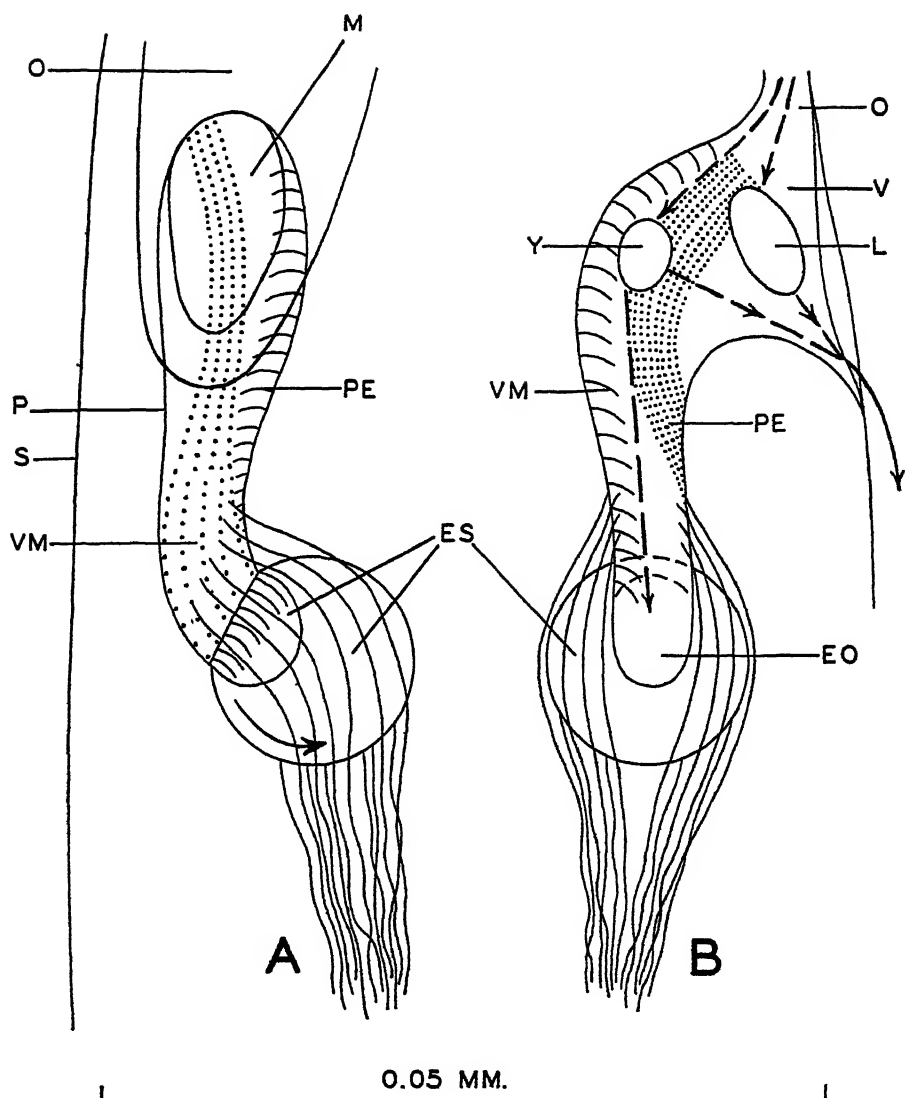


FIGURE 2. Camera outline showing the structure of the feeding apparatus in *Paramecium aurelia*. *A* and *B*, two views, one at right angles to the other. *O*, oral groove; *V*, vestibulum; *M*, mouth; *P*, pharynx; *EO*, esophageal opening; *ES*, esophageal sac; *EF*, esophageal fibers; *PE*, penniculus; *VM*, "Vierermembran"; *Y*, yeast-cell; *L*, a large particle; *S*, surface of the body; broken lines, paths taken by particles during the process of feeding; arrows, direction of movement.

The cilia which project into the esophageal sac from the end of the pharynx are doubtless part of the "Vierermembran." There are, of course, many more than are represented.

"Frequently in stained animals they are wrapped almost around the food vacuoles. In the living animal they can be observed only a short way beyond the gullet, but certainly not all in one plane and are capable of some movement. All morphological evidence points to a lax condition of these fibrils, with their distal ends free." He calls them "postesophageal fibrils".

Lund maintains that there are also "five or more heavy fibrils" which extend from their attachment at the anterior edge of the opening at the distal end of the pharynx, to the posterior edge where each ends in a "large granule" but that they can not be seen in living specimens. The larger granules, Lund thinks, are part of the neuromotor system. He calls these fibers "paraesophageal fibrils".

I have made extensive observations on numerous living specimens of the four species of *Paramecium* listed above, concerning fibers attached to the pharynx. Some of the observations were made on specimens which had been greatly compressed under the cover-glass, others on specimens stained with various vital dyes and still others on specimens in various stages of inanition. All were made with the highest grades of optical and illuminating systems (Mast and Bowen, 1944).

I could not see any fibers across the distal opening of the pharynx in any of the specimens studied, but in a few I could see several fibers which appeared to extend posteriorly from this end. I could not, however, make out any details as to their attachment or their length. This indicates that the index of refraction of these fibers is very nearly the same as that of the cytoplasm in which they are imbedded and that in this respect they differ from those found in the Peritricha for in these organisms they can readily be seen in the living state (Mast, 1944; Mast and Bowen, 1944).

#### THE FORMATION OF THE FOOD-VACUOLE

Paramecia ordinarily do not ingest anything when they are swimming actively (Mast and Lashley, 1916). When they are at rest or are swimming slowly in contact with a solid surface, the oral cilia produce a current down the oral groove into the vestibulum. This current contains in suspension all sorts of small solid particles many of which are carried into the vestibulum, but most of these are immediately carried out again. The rest pass through the mouth into the pharynx; some of these are thrown back out again, the rest and some fluid pass through the pharynx into the esophageal sac. Here the fluid with the particles in suspension usually rotates rapidly, and as more and more substance enters the sac, the particles in it become more concentrated and the sac becomes larger, and finally a portion of it separates from the pharynx as a full-fledged food-vacuole. All these phenomena can be seen readily and there is little disagreement concerning them; there is, however, marked diversity of opinion concerning the processes involved. These will be considered in detail under several headings in the following pages.

##### *Selection of particles.*

Metchnikow (1907, 1912) concludes that while paramecia ingest all sorts of small particles they take more of those which are digestible than of those which are not. This conclusion is supported by Bozler (1924), Losina-Losinsky (1931), Bragg (1936a, 1939), and others. It is consequently fairly certain that paramecia can differentiate between various small particles. There is, however, little known concerning the factors involved.

Bozler (1924) maintains that selection is usually made at or near the entrance to the pharynx by the response of individual cilia to contact with particles which have been carried into the vestibulum by the oral current, i.e., that if a particle comes in contact with a cilium, the cilium responds either by throwing the particle into the pharynx or into the outgoing current, depending upon the physical characters of the particle. He says (p. 189): "Man ersieht . . . dass das Hineinbefördern der Nahrungsteilchen nicht durch ein planmässiges Zusammenwirken der Cilien geschieht, sondern dass jede Cilie auf Grund der Reize reagiert, die sie direkt vom dem Nahrungskörper erhält." He maintains, however, that particles which have entered the pharynx are sometimes forced back out, even after they have nearly reached the distal end, but that this occurs only if the particles are large enough to fill the lumen of the pharynx. Bozler's contention that selection in *Paramecium* is dependent upon physical properties is supported by Schaeffer (1910) in observations on *Stentor*. Nelson (1933), on the contrary, presents convincing evidence that selection, in at least some ciliates, is dependent upon chemical properties.

Losina-Losinsky (1931) holds that discrimination between particles depends upon the action of cilia controlled by a central coordinating system, rather than upon the direct response of individual cilia to contact with the particles and that the stimulating agent is chemical rather than physical. He holds that acceptance of a particle is essentially the result of a chemopositive response and rejection to that of a chemonegative response.

I made numerous observations on the movements of particles in the feeding apparatus of *Paramecium caudatum*, *P. nucleatum*, and *P. aurelia*. Most of these observations were made under the oil-immersion objective with specimens variously oriented (Fig. 1). Some of the specimens were in culture fluid which contained numerous bacteria and various other solid particles and others were in culture fluid to which chinese ink, carmine, wheat starch and yeast-cells stained with congo red had been added respectively. No difference was observed in the three species studied.

When the paramecia were actively feeding, many particles of all sorts found in the surrounding medium were carried into the vestibulum, but only a small proportion of these passed on through the mouth into the pharynx. The rest passed out again—some immediately, some not until after they had circulated around in the vestibulum for a time. Nearly all the particles which entered the pharynx were small. A few of those and all the larger ones which entered, passed rapidly on into the esophageal sac without any indication of retardation on the way. The rest immediately after they had passed through the mouth, plunged, one at a time, into the band of cilia (the "Vierermembran") on the the aboral wall of the pharynx. Here they stopped a moment and then either passed back out through the mouth and the vestibulum or directly on into the esophageal sac (Fig. 2B).

It consequently seems to be the cilia in the "Vierermembran" near the proximal end of the pharynx which, after momentary contact, either throw the particles back out of the pharynx or down toward the esophageal sac. I have no evidence which indicates what it is that decides the fate of these particles after they have come in contact with these cilia. I have seen many stained, dead yeast-cells of all shapes and sizes rejected after they had come in contact with them and I have seen many of the same shapes and sizes accepted. There appeared to be neither rhyme nor reason in the process under these conditions.

The results presented indicate, therefore, that selection among the larger particles takes place in the vestibulum and selection among the smaller ones in the proximal region of the pharynx and the vestibulum; but they do not illuminate the problem concerning the nature of the stimulating agent involved in the selection of either.

Some of the particles ingested were surprisingly large. Several specimens of *Chilomonas paramecium*, 18  $\mu$  long and 10  $\mu$  wide, and starch grains, 16  $\mu$  in diameter, were seen to pass through the pharynx into the esophageal sac (Fig. 2). Bozler (1924) also found that paramecia sometimes ingest extraordinarily large particles. He found starch grains 11  $\mu$  in diameter in the food-vacuoles. The ingestion of particles as large as these requires great extension of the pharynx, for it usually is, in the smallest region, only five to seven micra in diameter. This shows that the pharynx is far from being as rigid as it appears to be.

Bills (1922) and Bozler (1924) maintain that particles are often rejected after they have passed well on down the pharynx, sometimes nearly, if not quite, to the esophageal sac. They consequently imply that selection of food may take place anywhere in the pharynx. Lund (1933, 1941) seems to hold that the particles which have entered the pharynx collect in front of a sort of fibrous screen at the mouth of the esophageal sac and then gradually slip through "one by one" into it and that selection takes place here, at least in part.

I have seen hundreds of particles of all sorts pass through the pharynx into the esophageal sac under various conditions, but I have never seen one rejected after it had passed beyond the first bend in the pharynx; nor have I ever seen any indication of decrease in the rate of movement between this bend and the sac.

*The enlargement of the esophageal sac and the increase in the concentration of particles in it.*

When a food-vacuole forms by constriction of the esophageal sac, a portion of the sac remains as a membrane over the distal opening of the pharynx. This membrane bulges slightly out into the cytoplasm and thus forms a shallow new esophageal sac. If there is active ingestion, this shallow sac rapidly becomes deeper and finally nearly spherical in form. It contains relatively much fluid and few particles at first, but as it increases in size the concentration of particles increases (at first slowly then more and more rapidly) until the sac often appears to be almost filled with them. The long cilia which extend from the end of the pharynx into the sac beat vigorously; this causes the particles in the sac to vibrate actively and its entire content to rotate. Rotation usually continues until the sac is closed; but sometimes it ceases long before the sac is closed and then only those particles vibrate which are in close contact with the cilia at the mouth of the sac. This is doubtless due to increase in the viscosity of the fluid in the sac. The quantitative relation between fluid and solid particles in the newly formed food-vacuole varies very greatly. Under some conditions the vacuole appears to be entirely filled with fluid, no particles whatever being visible even under the highest magnification, while under others, as stated above, it appears to be almost entirely filled with particles.

Bütschli (1889) and Horning (1926) contend that the content of the pharynx is in direct contact with the cytoplasm, i.e., that no membrane intervenes at the distal opening and that the droplets (vacuoles) which pass from the pharynx into the

cytoplasm are surrounded merely by a surface film. Bütschli seems to think that this continues, but Horning holds that in the cytoplasm, membranes form at the surfaces of the droplets. Gelei (1934) concludes that the content of the pharynx is separated from the cytoplasm by a definite membrane which persists as the esophageal sac enlarges, and that this membrane is porous; for he observed in stained whole mounts, that the cytoplasm adjoining it was strongly stained, whereas that adjoining the pellicle at the surface of the body and that adjoining the wall of the pharynx was only slightly stained, if at all.

Nirenstein (1905) maintains that the cytoplasm exerts suction ("eine Art Saugwirkung") on the esophageal sac, and that this causes the sac to enlarge very rapidly to its maximum size and fill with fluid containing but few particles, and he thinks that the cilia in the pharynx then force more particles into the sac and that this causes the observed increase in their concentration. That is, he seems to hold that the cilia come in direct contact with the particles and force them through the fluid in the pharynx and finally into the sac.

Bütschli (1889), Bozler (1924) and others maintain that the enlargement of the esophageal sac is due to pressure of fluid forced into it by the action of the pharyngeal cilia. They consequently do not agree with Nirenstein in reference to the cause of the enlargement, but they appear to agree with him in reference to the process involved in the concentration of particles. Bozler describes this as follows: A drop consisting largely of water ("ein Wassertropfen") is first formed; then solid particles are forced into it by the cilia in the pharynx ("hereingestrudelt") and whirled about by those which project from the end of the pharynx into the drop. After the drop has become nearly filled with particles these cilia rotate its content and press the particles together so as to get as many in as possible.

All the investigators referred to obviously hold that when the esophageal sac begins to enlarge almost nothing but water enters, that this continues until it has nearly, if not entirely, reached maximum size and that after this almost nothing but solid particles enter. This would account for the observed increase in the concentration of solid particles in the esophageal sac, but it is difficult to understand why the cilia in the pharynx should at first transport mainly water and later mainly solid particles.

Lund (1933) agrees with Bütschli and Bozler in reference to the cause of the enlargement of the esophageal sac, but he thinks that "paraesophageal fibrils" at the mouth of the sac are involved in the concentration of particles in it. He says (1933, p. 54): "Food material collects as it reaches these fibrils, so it is probable that they too aid in the concentration of food particles into the vacuoles" and (1941, p. 564) "Here particles of all sizes are trapped in the paraesophageal fibrils, while most of the fluid material presumably circulates back into the pharyngeal cavity. One by one the particles of food slip through the paraesophageal fibrils, and arrive in a growing bulge (the future food vacuole) on the dorsal side of the pharynx."

Lund found in section of paramecia, some particles which appeared to have been entangled in fibrils at the mouth of the esophageal sac. This led to the conclusions quoted above. I have, however, as previously stated, been unable to see in living paramecia any indication of an aggregation of particles at the mouth of the sac or cessation of movement of individual particles in this region. Nor have I been able to find any indication of backward circulation of fluid in the pharynx. The

particles observed by Lund among fibrils at or near the mouth of the sac may well have been deposited there by the microtome knife in sectioning the organisms.

It seems to me to be perfectly obvious that the cilia in the pharynx force fluid with particles in suspension into the esophageal sac and that it is this that causes the enlargement of the sac as Bütschli, Bozler and others maintain. But I could not observe any change in the concentration of the particles in the fluid forced into the sac, during its enlargement, i.e., I did not obtain any evidence indicating that the concentration was at first low and later high as Bozler's view demands. What then causes the observed increase in concentration?

### *Discussion*

Nirenstein (1905) maintains that after the food-vacuole has been formed and has left the pharynx it rapidly decreases in size and that this is due to loss of fluid. These contentions have been abundantly confirmed. The membrane around the food-vacuole is therefore pervious to water, and the loss of water, which causes the vacuole to decrease in size, is doubtless due to excessive external osmotic concentration and inward pressure of the stretched vacuolar membrane (Mast and Bowen, 1944).

If this is true, water must be continuously forced out of the esophageal sac during its enlargement, i.e., during the formation of the food-vacuole, for the factors involved in the loss of water from the fully formed food-vacuole function continuously during its formation. And since no solid particles leave during this time, it is obvious that this would result in increase in their concentration in the forming vacuole. But it does not account for the apparent observed increase in the rate of their concentration as the sac enlarges.

The difference between internal and external osmotic concentration is doubtless practically constant during the enlargement of the esophageal sac, as is also the pressure of the fluid against its inner surface, caused by the action of the cilia in the pharynx. The rate of outflow of water *per unit area* of membrane at the surface of the sac (provided there is no change in its permeability) is therefore practically constant; but since this area increases as the sac increases in size the rate at which water leaves the sac, i.e., the volume per second, also increases. Therefore, since as the sac increases in size the rate of inflow of fluid and particles through the pharynx remains practically constant, while the rate of outflow of fluid without particles increases, the rate of concentration of particles in the fluid in the esophageal sac must increase as the sac enlarges. Moreover, as the esophageal sac enlarges the membrane at its surface is continuously stretched, and as it is stretched it must be continuously built up by the interaction between substances in the sac and the adjoining cytoplasm so as to prevent rupture. It may well be, however, that the membrane thus formed becomes more and more pervious to water as it is stretched during the enlargement of the sac. If this is true, it also causes an increase in the rate of concentration of particles in the sac.

The conclusion that water continuously passes from the esophageal sac into the cytoplasm has an important bearing on various views concerning the origin of the water which is excreted by the contractile vacuoles. Eisenberg (1925) and Frisch (1937) hold that all this water enters the body through the pharynx; Bozler (1924), Fortner (1926), and Müller (1932) conclude that some of it enters through the

pellicle, and Kitching (1934, 1936, 1938) assumes, in some of his experiments, that all enters through the pellicle.

It has been demonstrated by several investigators that the total volume of the food-vacuoles (at maximum size) formed in a given period of time is only about one-third as great as the total volume of the contractile vacuoles (at maximum size) formed during the same length of time. This seems to prove that water enters the body through the pellicle as well as through the pharynx. Eisenberg, Frisch and others maintain, however, that some of the water that enters the pharynx passes directly into the cytoplasm, i.e., without entering the food-vacuole at all, and that this accounts for the observed difference between the total volume of the food-vacuoles and that of the contractile vacuoles. The evidence presented above that water continuously passes from the esophageal sac into the cytoplasm during the formation of the food-vacuoles, strongly supports the contention that the water excreted by the contractile vacuoles enters the pharynx; for the formation of the food-vacuole requires sufficient time to permit, during its formation, the passage of more water from it into the cytoplasm than passes from the food-vacuole into the cytoplasm after the vacuole has been fully formed.

In view of these and other considerations it is difficult to understand how Kitching, in the experiments referred to above, came to assume that all the water that was excreted by the contractile vacuole entered the body through the pellicle.

#### THE SEPARATION OF THE FOOD-VACUOLE FROM THE PHARYNX AND ITS MOVEMENT THROUGH THE BODY

There has been for many years much speculation concerning the processes involved in the separation of the food-vacuole from the pharynx and its movements after it has become free, but these problems still remain unsolved.

I have closely observed under the oil-immersion objective, numerous food-vacuoles leave the pharynx and pass through the body in each of the four species of *Paramecium* listed above, especially the first three. The specimens studied were under many different environmental conditions, both natural and artificial. Extensive variations were seen in each species but the results as a whole seem to show that the processes involved in the closing of the esophageal sac, the separation from the pharynx of the food-vacuole thus formed, and its passage through the body are fundamentally identical in the four species.

The following usually occurs: The esophageal sac enlarges and becomes nearly spherical with the major axis directed backward. The substance in the sac rotates strongly and the particles in suspension vibrate vigorously and become much concentrated. After the sac has become about twice as wide as the pharynx, it slides posteriorly from the diagonal end of the pharynx and becomes definitely pear-shaped, a nipple being drawn out on the vacuole as it leaves the pharynx. (No change whatever in the size of the end of the pharynx can be seen during this process.) A small portion of the esophageal sac remains as a membrane over the opening in the pharynx. This bulges out into the cytoplasm slightly, forming a shallow sac which soon enlarges to form a new vacuole (Fig. 3). After the nipple which connects the newly formed food-vacuole with the tip of the diagonal end of the pharynx has broken, the vacuole moves rapidly *through* the cytoplasm nearly to the posterior end of the body, and on the way it creates marked currents in the



cytoplasm, turns through approximately  $270^\circ$  and becomes spherical. After it has reached the end of this course, it stops a few moments, then slowly passes forward near the aboral surface to the anterior end of the body or part way, and then back along the opposite surface, to the anus.

As stated above, there is great variation in these phenomena. Some of these are set forth in the following paragraph:

During the separation of the food-vacuole from the pharynx, the cytoplasm around it ordinarily flows slowly backward and appears to elongate the vacuole slightly, but sometimes this cytoplasm does not flow at all and occasionally it flows so violently that it appears to elongate the vacuole greatly and actually pull it from

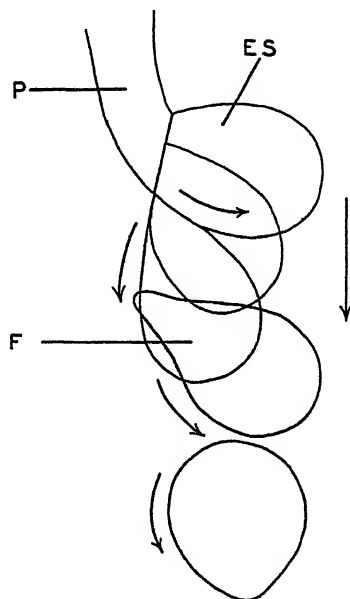


FIGURE 3. Camera outline of a portion of the feeding apparatus and some sketches illustrating the movement of the food-vacuole during its separation from the pharynx in *Paramecium aurelia*. P, pharynx; ES, esophageal sac; F, food-vacuole; arrows, direction of movement.

the pharynx. After the vacuole has been separated from the pharynx it almost invariably moves toward the posterior end of the body much faster than the surrounding cytoplasm, but occasionally it moves so slowly that it appears to be carried by the cytoplasm. On its way toward the posterior end of the body the vacuole usually turns somewhat more than half-way around but sometimes it does not turn at all, and sometimes it turns entirely around and occasionally even more than once; and it sometimes goes only a short distance on its usual course toward the posterior end of the body and is then carried forward. The vacuole usually becomes spherical before it has reached the posterior end of the body, but it sometimes does not become spherical until much later, if at all. This appears to be closely correlated with the viscosity of its content. Details concerning these phenomena are given in the next section (see also page 55-57).

*Discussion*

It is held by all who have investigated the subject that after the food-vacuole has reached the posterior end of the body, it is carried over the rest of its course in the cytoplasmic stream, i.e., by cyclosis, but there is marked diversity of opinion concerning the processes involved in the separation of the food-vacuole from the pharynx and its rapid movement toward the posterior end of the body.

Stein (1859), Bütschli (1889) and Bragg (1935, 1936) conclude that contraction of the distal end of the pharynx and cyclosis are involved. However, since no decrease in the size of the pharynx can be seen and the vacuole sometimes leaves the pharynx and moves away in the total absence of cyclosis, neither of these two factors is essential.

Nirenstein (1905) maintains that the cytoplasm adjoining the esophageal sac near the distal end of the pharynx contracts and separates the content of the sac from that in the pharynx and that the vacuole thus formed is carried away by cyclosis. Bozler (1924) supports Nirenstein in reference to the separation of the vacuoles from the pharynx, but he holds that the vacuole is not carried off by cyclosis. He contends that there is periodic backward streaming of the cytoplasm (which is not cyclosis) adjoining a group of fibers ("Schlundfaden") which extend from the pharynx nearly to the posterior end of the body and that the vacuole is carried posteriorly in this stream.

If the constriction in the esophageal sac is caused by the adjoining cytoplasm, one would expect to find a differentiated structure in the cytoplasm, i.e., a sort of sphincter. Nothing of this sort has, however, been found; and Bozler's contention that there is a stream of cytoplasm backward along one side of a bundle of fibers has not been confirmed. It is consequently not likely that the formation and movement of the vacuole is dependent upon either of these two postulated factors.

Kalmus (1931) and others seem to think that surface tension plays a predominant role in the formation of food-vacuoles, somewhat as it does in the formation of drops of water in the air. The formation of drops of water in air by the action of surface tension is, however, dependent upon weight. But since food-vacuoles suspended in cytoplasm have no weight, surface tension cannot be essential in their formation.

Gelei (1934) says the food-vacuoles are torn from the pharynx, but he does not say by what means.

Lund (1941) concludes that the food-vacuole is separated from the pharynx and forced toward the posterior end of the body by the action of the "postesophageal fibrils". He says (p. 564): "With the growth of the vacuole the postesophageal fibrils contract about its base, the vacuole is pressed posteriorly, and once it is released the fibrils conduct it into the cytoplasm with considerable rapidity. Their concerted action produces an effect somewhat resembling that produced by a peristaltic wave in the esophagus of higher vertebrates."

Lund did not actually see the processes described nor could I see anything of the sort. I believe, however, that his postulations are eminently sound, for they reasonably account for nearly all that has been seen. There is, however, one observation that seems to require further elucidation.

Nirenstein (1905, p. 451) maintains that if the food-vacuole is directly over or under the diagonal opening of the pharynx which now appears as a clear oval

("heller Oval"), it can be seen that as the vacuole leaves the pharynx, the minor axis of the oval decreases until the oval becomes a slit ("ein heller Spalt") before the major axis decreases. I could not see these phenomena, but the observations are so difficult that this casts no reflections on their validity. Nirenstein thinks that the decrease in the minor axis, before the major axis decreases, must be due to contraction of the cytoplasm ("Plasma") adjoining the esophageal sac near its connection with the pharynx. Lund asserts, as previously stated, that there are several fibers which extend over the opening at the distal end of the pharynx, i.e., the mouth of the esophageal sac. I have already presented evidence which shows that if these fibers exist (and Lund's figures seem to prove that they do), they are not inside of the pharynx or the sac. They must, therefore, be outside, some doubtless being on either side of the sac close to the end of the pharynx. If this is true, these two groups of fibers become much separated in the middle and stretched as the sac enlarges. And if they now contract the opening of the sac will be laterally squeezed and consequently will become slit-like, i.e., it will assume a form which is in accord with Nirenstein's contention. It may well be, therefore, that these fibers (called "paraesophageal fibrils" by Lund) function in the separation of the food-vacuole from the pharynx.

#### INITIATION OF THE SEPARATION OF THE FOOD-VACUOLE FROM THE PHARYNX

The earlier investigators held that in *Paramecium* the size of the food-vacuole controls the initiation of its separation from the pharynx, and this idea seems still to be widely held. For example, Bragg (1935) says the food-vacuole "grows gradually larger till, reaching its full size, it suddenly drops into the endoplasm." This contention is supported by the fact that in a given individual successive vacuoles are usually nearly the same in size, but it does not account for the great difference in their size often observed.

Bozler (1924) asserts that the separation of the food-vacuole from the pharynx is initiated by contact of particles with the inner surface of the vacuolar membrane. He bases this assertion on his contention that paramecia will not form food-vacuoles in a medium which does not contain particles. In this contention he does not, however, agree with Schewiakoff (1894), who maintains that particles are not necessary. Bragg (1935) says that in *Paramecium trichium* contact of large particles with the inner surface of the vacuolar membrane is always immediately followed by separation of the vacuole from the pharynx. This seems to support Bozler's contention. Bragg says however that many of the food-vacuoles formed in this species have no large particles and that in *Paramecium caudatum* large particles in the vacuole have no effect on their separation from the pharynx. He consequently concludes that they are not necessary.

I made many observations concerning this on each of the four species of *Paramecium* under consideration and found in all these species that the food-vacuole frequently leaves the pharynx immediately after a large particle has entered but that this does not always occur in any of them. I observed that when *Paramecium trichium* is ingesting yeast-cells, the food-vacuole usually leaves the pharynx immediately after one of these cells has entered but that occasionally it does not leave until after two have entered. I also found that this obtains for each of the three other species studied, when they are ingesting specimens of *Chilomonas paramecium*, and in these

I observed that occasionally the vacuole did not leave the pharynx until after three had entered.

These results are therefore not in full accord with Bragg's contentions. However, I also observed in all the species, food-vacuoles which had no large particles whatever. This supports Bragg's conclusion that contact of large particles with the vacuolar membrane is not necessary for the separation of the vacuole from the pharynx. This conclusion is also supported by the fact that I have under various conditions seen many food-vacuoles form in which no particles whatever could be seen. It is therefore very probable that in Bozler's observations the failure of the paramecia to form food-vacuoles in a particle-free medium was due to injurious action of the medium he used rather than to the absence of particles in it.

The evidence in hand obviously throws but little light on the processes involved in the initiation of the separation of the food-vacuole from the pharynx in paramecia, and this is also true for other forms. Mast and Bowen (1944) considered this problem theoretically in reference to the Peritricha and reached the following conclusions: "It is highly probable that waves start at fairly definite intervals in the pharyngeal ring and pass simultaneously down all the esophageal fibers and that each of these sets of waves initiates a constriction in the esophageal sac, if it contains sufficient fluid to make a constriction possible. If this is true, the size of the vacuole is correlated with the rate at which the fluid is forced into the esophageal sac by the cilia in the pharynx and the rate at which it leaves this sac by osmosis. If these processes and the interval between successive waves depend upon the composition of the surrounding fluid, the temperature and the physiological state of the organism, it accounts for the observed variation in the size of the food-vacuoles and the intervals between their formation." It seems to me that these conclusions apply equally well to *Paramecium*. Moreover, Bozler (1924) says that in this genus a momentary current passes posteriorly along the "Schlundfaden" at about 50-second intervals. These currents, he asserts, are entirely independent of cyclosis. I have seen similar currents in this region, though not very distinctly. I believe they are produced by periodic action of the esophageal fibers. If this is true, it supports the conclusions presented above.

#### SIZE, RATE OF FORMATION, AND SHAPE OF THE FOOD-VACUOLE

##### *Size*

It is well known that the food-vacuoles formed in *Paramecium* vary greatly in size, but very little is known concerning the cause of this variation.

Metchnikow (1912) maintains that if paramecia are transferred from a solution which is poor to one which is rich in digestible substance (i.e., bacteria, milk, egg-yolk, etc.) the first vacuole formed is always huge, judging from his figures, nearly 40 times larger than normal vacuoles. He also maintains that in solutions which contain only indigestible particles (carmine, chinese ink, etc.) the food-vacuoles formed are abnormally small. He does not designate the species studied, but his figures indicate that it probably was *aurelia*.

I repeated Metchnikow's experiments and obtained results which on the whole support his contention; but I found that the size of the vacuoles is not at all closely correlated with the composition of the surrounding medium. That is, I found that

the extent of change in the size of the vacuoles formed after transfer to a given medium varied greatly.

Dogiel et al. (1927) made observations on the food-vacuoles formed by paramecia<sup>4</sup> in solutions of salts, containing numerous particles of chinese ink in suspension. They found that in solutions of  $MgCl_2$ ,  $MgSO_4$ , and  $FeSO_4$ , the food-vacuoles formed are long, tubular and coiled ("Nahrungsschlauche") but that in solutions of  $BaCl_2$  they are minute and spindle-shaped. Judging from the sketches presented, the former would average about  $60\ \mu$  in diameter in spherical form, and the latter only about  $4\ \mu$ . Both were filled with particles of ink.

I repeated these experiments on each of the four species of *Paramecium* listed above, with each of four brands of chinese ink in a much greater range of concentrations of the salts than was used by Dogiel et al. Moreover, the solutions of all the salts were made up respectively in distilled water, tap-water, and fluid taken from the cultures. The paramecia ingested the particles of ink freely in all the salt concentrations tested, except those which were so high that they were injurious; but I observed no indication of the formation of abnormal food-vacuoles in any of the solutions, either in form or in size.

Dogiel et al. assume that the abnormal food-vacuoles they observed were due to the action of the salts used. They say, however, that they did not obtain such vacuoles if the chinese ink was omitted. This, and the fact that I obtained no abnormal vacuoles, strongly indicates that the abnormality observed by them was due to the action of the ink they used rather than the salts.

Cosmovici (1931) maintains that "*Colpidium colpoda*" in culture fluid containing "amylodextrine" forms tubular food-vacuoles, some of which extend from the pharynx to the anus. He concludes that there is in the ciliates a very complicated closed capillary digestive system, through which substance is moved by waves of cytoplasmic contraction, and that cyclosis is an optical illusion, due to this movement. Amazing conclusions!

I repeated and extended Cosmovici's observations using paramecia in place of colpidia, but obtained no evidence whatever in support of his contentions.

Frisch (1937) made very extensive measurements on food-vacuoles in specimens of *Paramecium nucleatum* taken from given cultures on successive days for three weeks or more. The cultures remained in a flourishing condition for nearly two weeks and then declined. During the flourishing period the average diameter of the food-vacuoles formed in different individuals varied from  $17.25\ \mu$  to  $25\ \mu$  with  $65.55\ \mu$  for the largest vacuole measured. As the cultures declined the food-vacuoles formed decreased in diameter "first to 13.80 microns, then progressively to 10.35, 6.90 and 3.45 microns." The paramecia were plump and ranged from  $227\ \mu$  to  $330\ \mu$  in length until the cultures began to decline, then they gradually became thin and somewhat shorter. It is consequently evident that in these cultures the size of the food-vacuoles formed varied directly with the size of the paramecia. Frisch maintains, however, that in well-fed paramecia the size of the food-vacuoles is not closely, if at all, correlated with the size of the body and that the observed decrease in the size of the food-vacuoles during the decline of the cultures was largely if not entirely, due to decrease in quantity and quality of food, i.e., bacteria.

<sup>4</sup> The species is not designated but the size of the paramecia calculated from the sketches presented and the fact that only one micronucleus is figured, indicate that it was *caudatum*.

I repeated the observations made by Frisch and extended them to other species. I also made observations on the effect of increase in the viscosity of the surrounding fluid. The results obtained are in full accord with those obtained by Frisch. I did find, however, that while within a given species, the size of the food-vacuoles does not appear to be correlated with the size of the individuals, the vacuoles formed by the two smaller species (*aurelia* and *trichium*) were, in general, much smaller than those formed by the two larger (*caudatum* and *nucleatum*) and that the size in all the species appears to depend upon the viscosity of the surrounding medium.

In the observations on the effect of viscosity, polyvinyl alcohol or methyl cellulose was added to culture fluid containing paramecia on a slide. It was found that if the viscosity of the fluid became high enough to retard locomotion, but not high enough to inhibit it, the paramecia ingested the fluid rapidly, formed unusually large food-vacuoles and soon became well filled with them (Fig. 4). In some of these vacuoles a considerable number of solid particles could be seen but in others none

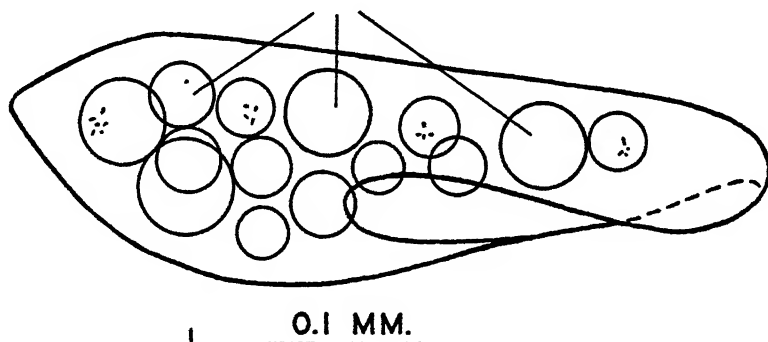


FIGURE 4. Camera outline of *Paramecium caudatum* showing food-vacuoles formed in a viscous solution of polyvinyl alcohol. F, food-vacuoles. The small dots in the food-vacuoles represent solid particles. Most of the vacuoles did not contain any.

whatever, despite thorough examination of much flattened specimens under the oil-immersion objective. Bozler (1924) obtained somewhat similar results with other viscous substances.

Lee (1942) asserts that "preliminary observations" show that the size of the food-vacuoles in *Paramecium* is independent of the hydrogen-ion concentration of the surrounding medium, but he gives no details regarding the results of the observations made. It may well be, therefore, that this assertion is not strictly true. However this may be, the evidence in hand seems to show that there are at least four environmental factors which are involved in the control of the size of the food-vacuoles, namely, the quantity and the quality of the particles in suspension, and the chemical composition and the viscosity of the surrounding fluid. The question now arises as to how these factors function.

Mast and Bowen (1944) found in observations on *Vorticella similis* that the food-vacuoles vary *greatly* in size without any variation in the number or kind of particles suspended in the surrounding fluid or in its chemical composition. But

they did find that under certain conditions the size is definitely correlated with the quantitative rate of ingestion.

They postulate, as stated above, that the food-vacuole is formed by constriction in the esophageal sac caused by the action of the esophageal fibers and that this constrictive action occurs at fairly regular intervals regardless of the size of the esophageal sac. If this is true, it is obvious that if the constrictive action occurs when the sac is large, a large vacuole will be formed and that if it occurs when the sac is small, a small vacuole will be formed; and it is also obvious that if the quantitative rate of ingestion is high the esophageal sac will become, during the interval between successive constrictive actions, larger than if it is low. But the size of the sac will also depend upon the quantitative rate of passage of fluid from the sac through its surface membrane into the adjoining cytoplasm. According to this view then the primary factors involved in controlling the size of the food-vacuoles are (1) the quantitative rate of ingestion of fluid and solid particles, (2) the quantitative rate of passage of fluid from the esophageal sac into the cytoplasm, and (3) the length of the intervals between consecutive constrictive actions of the esophageal fibers. If this obtains all other factors that may be involved (the quantity and the quality of particles in the surrounding fluid, the chemical composition of this fluid, the physiological state of the organisms, etc.) function by modifying the primary factors.

This hypothesis is in full accord with the facts in hand, concerning the control of the size of the food-vacuoles in the Peritricha, and it also appears to account for all that is known concerning this phenomenon in *Paramecium*.

#### *Rate of formation*

Metchnikow (1912) contends that the rate of formation of food-vacuoles is dependent upon the hydrogen-ion concentration and upon the temperature of the surrounding fluid but that it is not dependent upon the number of particles suspended in it.

Bozler (1924) asserts that the formation of a food-vacuole requires 4 to 5 minutes if the particles in suspension are scarce and only 1 to 2 minutes if they are abundant. He consequently does not agree with Metchnikow in this respect.

Frisch (1937) seems to agree with Bozler in reference to the correlation between the concentration of particles and the rate of formation of food-vacuoles. He found that the time required for the formation of the food-vacuoles measured in the observations described above, varied from 17 to 365 seconds, and he maintains that the time required for the formation of a food-vacuole is not dependent upon the size of the vacuole but that it is largely, if not entirely, dependent upon the quantity and the quality of the food present. He writes: "This variation did not depend upon the size of the food-vacuole formed but probably upon the quantity and the quality of bacteria present in the immediate vicinity of the animals." He does not elucidate this dependence, but he doubtless holds that the time varies inversely with the quantity of bacteria and the extent of their usefulness as food.

Lee (1942) demonstrated that the rate of formation of food-vacuoles is closely correlated with the hydrogen-ion concentration and the temperature of the surrounding fluid. He consequently supports Metchnikow.

No definite views have been expressed as to how the factors involved act in the

control of the rate of formation of food-vacuoles. Lee implies, however, that the rate is directly proportional to the activity of the cilia in the vestibulum ("peristome") and that this is correlated with acidity, temperature, etc. This would account for the facts observed by Lee, but it would not account for the correlation described above between the rate of formation of vacuoles and the concentration of particles in suspension in the surrounding medium. Moreover, I have repeatedly seen paramecia in which the cilia in the vestibulum were very active and numerous particles entered the vestibulum, but none passed into the pharynx, all being thrown out. This indicates that the amount of substance which enters the pharynx depends upon the nature of the activity of the cilia in the vestibulum quite as much as upon the magnitude of the activity.

If the food-vacuoles are separated from the pharynx by constrictive actions of the esophageal fibers and these actions occur at regular intervals in accord with the hypothesis presented above, the rate of formation of the vacuoles must depend upon the length of these intervals; and the effect on the rate of formation produced by the factors considered above, and others (i.e., acidity, temperature, physiological states, etc.) must be due to alteration produced by them, in the length of the intervals.

### Shape

It is generally agreed that the newly formed food-vacuoles in *Paramecium* are usually nearly spherical but that they sometimes are distinctly spindle-shaped (Nirenstein, 1905; Bozler, 1924; Dunihue, 1931; Gelei, 1934; Bragg, 1935, 1936). There is no controversy concerning the cause of the spherical form, but there are several views as to the cause of the spindle-shape.

Nirenstein holds that the spindle-shape is due to constriction of the distal end of the forming vacuole by contraction of the adjoining cytoplasm and to drawing out of the proximal end to a point as the vacuole leaves the pharynx. Bozler maintains that the food-vacuole is pressed against a bundle of fibers ("Schlundfaden") on its way from the pharynx to the posterior end of the body and that this causes the spindle-shape. Gelei found that the portion of the esophageal sac which remains attached to the pharynx after a food-vacuole has left, is sometimes pointed and he maintains that this results in a point on the distal end of the following vacuole and consequently in a spindle-shape. Mast and Bowen (1944) found that this regularly occurs in the *Peritricha* in which the newly formed food-vacuole is always spindle-shaped. Their results consequently support Gelei's contention. Bragg (1936) concludes that the food-vacuole, immediately after it leaves the pharynx, is usually spindle-shaped in *Paramecium caudatum*, rarely in *P. nucleatum* and never in *P. aurelia* and *P. trichium*. He concludes that its form is a species characteristic, but he gives no information as to why, in a given species, it is sometimes nearly spherical and sometimes definitely spindle-shaped.

I found in observations on paramecia which had been in culture fluid containing neutral red in moderate concentration, that nearly all the food-vacuoles formed in *P. caudatum* and *P. nucleatum*, but none of those formed in *P. aurelia* and *P. trichium*, were spindle-shaped. I also found that, while in the spherical vacuoles there was invariably violent movement, in the spindle-shaped vacuoles there was little or none. This shows that the viscosity of the fluid in the vacuoles differs greatly and that the shape of the vacuoles is correlated with it.



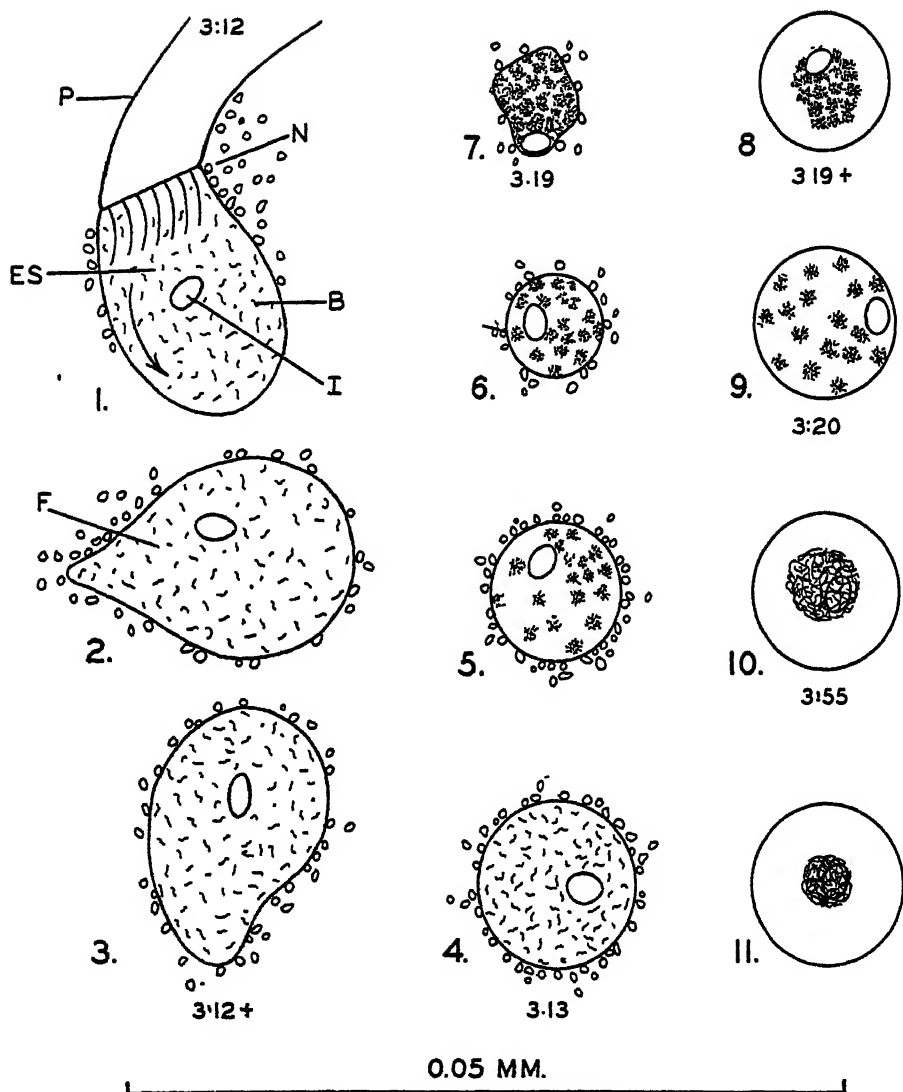


FIGURE 5. Camera outlines of a food-vacuole in a specimen of *Paramecium aurelia*, showing changes in form, size, and content, and changes in the position of neutral-red granules at the surface. *P*, pharynx; *ES*, esophageal sac; *F*, food-vacuole; *B*, bacteria; *I*, indigestible body; *N*, neutral-red granules; 3:12-3:55, time.

Note that the vacuole rapidly became spherical then decreased slowly but greatly in size, then increased rapidly and greatly. Note also that the numerous neutral-red granules at the surface of the vacuole all disappeared during its rapid enlargement and that the bacteria appear to have died and agglutinated during the decrease in size.

CHANGES IN SHAPE AND SIZE OF THE FOOD-VACUOLE AFTER IT HAS LEFT  
THE PHARYNX

Nirenstein (1905) contends that the food-vacuole in *Paramecium*, after it has left the pharynx, becomes spherical and decreases in size to about one-tenth and then increases until it is somewhat larger than it was originally. These contentions have been confirmed by others.

I made detailed observations concerning these phenomena in *Paramecium caudatum*, *P. nucleatum*, and *P. aurelia*. The results obtained show that they are fundamentally the same in these three species. The changes observed in a typical food-vacuole in *Paramecium caudatum* are illustrated in Figure 5.

This figure shows that the vacuole, after it had become free from the pharynx, rapidly became spherical and slowly decreased in size to nearly one-twentieth and then very rapidly increased until it was nearly as large as it had been originally. The figure indicates that as the vacuole decreased in size the bacteria in it died and aggregated in numerous small clumps, and that fluid passed out into the surrounding cytoplasm until there was practically none left, and the vacuole was so packed full of solid particles that the surface was very irregular. The figure indicates also that the numerous "neutral-red granules" at the surface of the vacuole when it left the pharynx, disappeared during its rapid enlargement. All these phenomena varied greatly in extent and time. For example, under some conditions there was no perceptible change in the size of the vacuoles at all. Details concerning some of them are given in the next section (see pages 55-57).

Mast and Bowen (1944) observed similar phenomena in the food-vacuoles in the Peritricha. They make the following statements in reference to the causes of the changes in size: "The loss of fluid, resulting in the decrease in the size, is probably due in part to difference in the osmotic concentration of fluids in the vacuoles and the cytoplasm<sup>5</sup> (that of the latter being higher than that of the former) and in part to inward pressure of the elastic membrane on the surface of the vacuoles, which was stretched by the pressure of fluid forced into them by the pharyngeal cilia. The inflow of fluid, resulting in increase in size, is probably entirely due to greater osmotic concentration within the vacuole than without. If this is true, the internal osmotic concentration must increase greatly during the time that the vacuole remains minimum in size. This could readily be brought about by transformation in the vacuole of osmotically inactive to osmotically active substance, for example, starch to sugar. In food-vacuoles which contain lactose, the gelatinous substance in them, referred to above, increases greatly in viscosity as the vacuoles decrease in size (as indicated by observations on Brownian movement) and then decreases greatly as they increase in size. The increase in viscosity is correlated with increase in acidity. It may well be that this increase in acidity causes chemical changes in the gelatinous substance which result in increase in osmotic concentration and that this

<sup>5</sup> This contention is strongly supported by the following observations: Mast and Bowen (1944) found that the food-vacuoles in *Vorticella similis* do not decrease in size if lactose in sufficient quantity is added to the culture fluid; and Fortner (1933) found the same in *Paramecium caudatum* in culture fluid to which numerous crushed bacteria had been added. In both of these observations the osmotic concentration of the fluid in the food-vacuoles was probably as high or higher than that of the surrounding cytoplasm, hence the retention of the water in them and no decrease in size.

in turn causes the rapid inflow of fluid from the cytoplasm which in turn, owing to decrease in acidity, causes the observed decrease in viscosity."

These statements are equally applicable to the food-vacuoles in *Paramecium*.

#### THE HYDROGEN-ION CONCENTRATION OF THE CONTENT OF THE FOOD-VACUOLE IN *PARAMECIUM*

It has been known for many years that after the food-vacuole in protozoa has been formed the hydrogen-ion concentration of its content increases considerably and then decreases again (Metchnikoff, 1889; LeDantec, 1890; Greenwood and Saunders, 1894; Nirenstein, 1905; et al.<sup>6</sup>). There is however marked diversity of opinion concerning the magnitude of these changes and the factors involved in producing them. Moreover, it has recently been maintained that in *Paramecium* the ingested substance becomes alkaline before the food-vacuole is formed, i.e., that there is in the vacuole a "preliminary alkaline phase."

##### *The preliminary alkaline phase of the food-vacuole*

Shipley and DeGaris (1925) made observations on specimens of *Paramecium caudatum* and *P. aurelia* in acid culture fluid to which phenol-sulfonphthalein (phenol red) had been added. They maintain that this fluid and the bacteria in it were distinctly yellow (acid), but that as it passed through the pharynx it became "faintly but distinctly pink . . . next to the wall of the pharynx", and that the forming food-vacuole was "filled with an alkaline [pink] fluid" which later became yellow (acid), and then pink (alkaline) again. They seem to hold that an alkaline substance is secreted by the wall of the pharynx.

Shapiro (1927) repeated the observations of Shipley and DeGaris and extended them to paramecia in neutral and in alkaline culture fluids. He says that in the neutral culture fluid "the vacuoles at first had a very light shade of pink" (alkaline), but that in the acid and the alkaline culture fluids there was no evidence of this. He concludes, however (p. 49), that "the observations made by Shipley and DeGaris of an alkaline stage at the beginning of the cycle has been verified." Dunihue (1931) asserts, on the contrary, that he was unable to confirm these observations.

I put into each of seven test-tubes a given quantity of fluid from a vigorous culture of *Paramecium caudatum* and added a different quantity of phenol red to the fluid in each, so as to produce a graded series of concentrations. All the solutions became distinctly yellow, and comparison with a series of buffers containing phenol red, showed that they were acid. After 24 hours the paramecia in the two strongest solutions were dead, but those in the rest of the solutions appeared to be in excellent condition. Some from each of these solutions were mounted and studied. They ingested fluid and bacteria in all, and the results obtained were the same in all.

<sup>6</sup>Engelmann (1879, p. 349) had previously observed ingested blue particles of litmus become red in *Paramecium aurelia*, *Stylonychia mytilus*, and *Amoeba diffuens*. But he did not know that this change in color was correlated with the content of the food-vacuoles. He assumed that the ingested particles of litmus were in direct contact with the cytoplasm and he therefore concluded that the observed change in their color demonstrated that the protoplasm is acid in these species.

Under a magnification of 400 (20 oculars and 20 objective) the stream of fluid in the pharynx was definitely yellow except a thin layer at the surface, i.e., a thin layer adjoining the wall of the pharynx, which was distinctly purplish pink. The fluid at the surface of the forming food-vacuole was also purplish pink, but that in the central portion was yellow, and the bacteria in it appeared to be the same in color as the fluid. After the vacuoles left the pharynx, they decreased slowly in size and the bacteria became intensely yellow, much deeper yellow than the fluid around them. Later the vacuoles increased rapidly in size, and the fluid in them became light purplish pink and the bacteria dark purplish pink.

These results are essentially in accord with the contentions of Shipley and DeGaris, and they appear to support their implication that the wall of the pharynx secretes an alkaline substance. However, on further examination of the paramecia in the culture fluids containing phenol red, I found that the surface of the content of the contractile vacuoles was also purplish pink, that is, that it was the same in color as the surface of the content of the pharynx, and I later observed the same purplish pink color in the pharynx and the contractile vacuoles in paramecia in culture fluid which did not contain any dye. Moreover, under a magnification of 1200 (20 oculars and 60 oil-immersion objective) there was no indication whatever of purplish or pinkish color in the pharynx or the contractile vacuoles, either in the paramecia in the culture fluid containing phenol red or in those in the culture fluid without any dye.

These results seem therefore to indicate that the purplish pink color observed in the lower magnification was due to refraction phenomena, not to change in acidity. It is consequently highly probable that the pinkish color observed in the pharynx and the forming food-vacuole by Shipley and DeGaris and Shapiro was not due to decrease in acidity.

I made numerous observations with several other indicator dyes, concerning a decrease in the acidity of the fluid in the pharynx in *Paramecium* but obtained no evidence of any. Moreover, Lund (1914) demonstrated fairly conclusively that in *Bursaria* there is a definite increase. That is, he found precisely the opposite from what Shipley and DeGaris maintain occurs in *Paramecium*. The evidence in support of a "preliminary alkaline phase" in the food-vacuoles in *Paramecium* is consequently negligible.

#### THE MAGNITUDE OF THE CHANGES IN THE HYDROGEN-ION CONCENTRATION IN THE FOOD-VACUOLE

Bozler (1924) was probably the first to attempt to measure the hydrogen-ion concentration of the content of the food-vacuole. He made observations on food-vacuoles in paramecia which had ingested yeast-cells stained with congo red; and he concluded that the hydrogen-ion concentration of their content increases approximately to pH 3. Nirenstein (1925) repeated and extended Bozler's observations and concluded that it increases nearly, if not quite, to pH 1. Neither of these investigators measured the subsequent decrease in hydrogen-ion concentration. Shapiro (1927) measured this and also the preceding changes. He made observations on paramecia in culture fluid containing respectively litmus, congo red, and phenol red, and concluded that in neutral culture fluid the acidity at first decreases to about pH 7.6 ("preliminary alkaline phase"), then increases to a maximum of

pH 4, and then decreases about to pH 7. Kalmus (1931) confirmed Nirenstein's conclusion.

I repeated and extended the preceding observations made with dyes and I also made observations on ingested crystals.

### *Ingested crystals*

Neutral-red crystals were produced and tested for solubility and changes in color in relation to hydrogen-ion concentration as described in preceding papers (Mast, 1942; Mast and Bowen, 1944). Some fragments of these were added respectively to neutral and slightly acid or alkaline culture fluid containing paramecia on a slide and covered with a cover-glass supported on ridges of vaseline.

In some tests the culture fluid was taken from vigorous cultures which contained numerous bacteria; in others it was taken from old cultures which contained only a few bacteria, and in still others it was taken from a fresh culture which contained very few bacteria. Starch was added to the culture fluid in some of the tests. Observations were made on three different species of paramecia, namely, *caudatum*, *nucleatum*, and *aurelia*. The results obtained are essentially the same for the three species studied.

The paramecia fed freely in all the solutions used. Some of the food-vacuoles formed contained, in addition to the culture fluid ingested, only bacteria, others chiefly starch, and still others fragments of crystals, bacteria, and starch in various proportions. The fragments of crystals ingested ranged in size from irregular masses not much larger than the bacteria, to needle-like structures nearly half as long as the diameter of the paramecia.

The largest fragments invariably punctured the wall of the vacuole and passed out into the cytoplasm. Some of these were continuously observed under the oil-immersion objective for an hour or more. During this time they circulated several times around the body in the cytoplasmic stream and were then ejected. A few "neutral-red granules" became attached to the surface but no perceptible change occurred in the crystals, either in color or in form.

All the smaller fragments of crystals ingested remained in the food-vacuoles. These in most of the vacuoles did not change perceptibly and were ejected intact with the rest of the undigested substance. But those in some changed in color from brownish yellow to pink, and some of these rounded up and became nearly spherical. I did not see any disintegrate completely; but the fact that they rounded up shows that they became plastic and that they probably had partially dissolved.

These changes invariably occurred soon after the vacuoles had left the pharynx, but not until after they had decreased considerably in size. They occurred in vacuoles which contained only a few bacteria as well as in those which contained many, but not in all, and they did not occur in any vacuoles which were well filled with starch grains and contained but little fluid, and consequently decreased but little in size.

In acetate buffer solutions the crystals became pink immediately at pH 5 and lower, and dissolved in a few seconds. At pH 5.6 they became pink at the surface in 2 to 3 minutes and dissolved in 15 to 60 minutes. In hydrochloric acid, pH 4, they became pink at the surface immediately and dissolved in 10 to 20 minutes, but at pH 5 it required several minutes for them to become pink and more than 60

minutes for all to dissolve. Neutral-red crystals appear therefore to be considerably more readily soluble in acetate buffer solutions than in solutions of hydrochloric acid in distilled water. This difference seems to be correlated with the difference in the amount of buffers present.

The fact that the crystals became pink and, at least partially, dissolved in a few minutes in some food-vacuoles and did not change perceptibly in others shows that the maximum hydrogen-ion concentration of the content of the vacuoles varies considerably. This variation seems to be, at least in part, due to difference in the extent of the decrease in size of the vacuoles, for there was, as stated above, no change in the crystals in any of the vacuoles until after the vacuoles had decreased considerably in size.

The fluid in the food-vacuoles is doubtless well buffered and consequently resembles acetate buffer solution in this respect. Therefore, the facts that in acetate buffer solution the crystals become pink at pH 5.6 and that in many of the food-vacuoles they do not become pink indicate that the hydrogen-ion concentration of the fluid in these vacuoles does not reach pH 5.6. The results presented below show, however, that the maximum acidity reached in other vacuoles is very much higher.

### *Ingested dyes*

The observations on ingested dyes were made as follows: A series of small test-tubes, containing some of the dye under consideration and appropriate buffer solution which differed by pH 0.2 in successive tubes, was prepared. Then culture fluid containing paramecia and bacteria was mounted between two parallel ridges of vaseline on a slide and dye or yeast-cells which had been stained by boiling in water containing dye, added. Then the preparation was covered with a cover-glass and the color of the content of the food-vacuoles formed, compared with that of the buffer in each of the test-tubes, and usually also with that of stained yeast-cells mounted respectively in buffer solution from each of the test-tubes. The hydrogen-ion concentration of the content of the food-vacuole was assumed to be that of the buffer and the stained yeast-cells in it, which it most nearly resembled.

### *Neutral red (pH 6.8, red—pH 8, auburn)*

Three species of *Paramecium* were studied, *caudatum*, *nucleatum*, and *aurelia*. The results obtained were essentially the same in these three species.

Owing to the activity of the paramecia, it was usually impossible to keep a given food-vacuole under observation for more than a few minutes, especially under high magnification. I succeeded however in continuously observing one vacuole under 20 oculars and a 60 oil-immersion objective throughout practically its entire existence. Since these observations are exceptional and the results obtained concern various important phenomena aside from changes in hydrogen-ion concentration, they will be presented in detail.

The paramecium in which this vacuole formed, had been for 24 hours in culture fluid containing numerous bacteria and neutral red in low concentration. When it was discovered there were scattered through the cytoplasm about a dozen food-vacuoles and numerous small red granules or droplets often called "neutral-red granules." The vacuoles varied greatly in size and color. Some were pinkish red,

others yellow and still others various shades between. The red granules were much more abundant in the region around the distal end of the pharynx than elsewhere. They were especially abundant near the distal end of the aboral surface of the pharynx and the surface of the forming food-vacuole. There were so many granules on these surfaces that they appeared to form a continuous layer. Some of the formed food-vacuoles were also well covered with granules but others had none at all.

The food-vacuole under observation began to form about 3:11 P.M. It contained numerous bacteria and a small yellow neutral-red crystal which served admirably to distinguish the vacuole from others in the body. The bacteria were swimming actively and the entire content of the vacuole rotated rapidly, owing to the action of the cilia which projected from the pharynx into it. The vacuole left the pharynx at 3:12 P.M. and passed very rapidly nearly to the posterior end of the body. It turned approximately through  $180^\circ$  on the way, created violent currents in the cytoplasm and dragged numerous neutral-red granules after it. It was pear-shaped when it left the pharynx but became nearly spherical on the way to the posterior end of the body, i.e., in about one-fourth second. The bacteria and the fluid in it now appeared to be distinctly pinkish in color, but this was due to the red granules on the surface, for careful focusing showed that both the fluid and the bacteria in it were colorless. By 3:12½ P.M. the vacuole had decreased about one-fifth in diameter and the crystal had become spherical and crimson in color, but the bacteria were still colorless and active and the fluid was also still colorless. The crystal had apparently partially dissolved and had become plastic. At 3:15 P.M. the bacteria were motionless and faintly pink in color, but the fluid was still colorless. No further change had occurred in the crystal and the surface of the vacuole was still well covered with neutral-red granules. Some of these granules were seen to leave and others to approach the surface. *None passed into the vacuole.* By 3:17 P.M. the vacuole had decreased nearly one-half in diameter and had moved forward considerably. The bacteria had become deep pink in color, but no perceptible change had occurred in the fluid or the crystal or in the number of neutral-red granules on the surface. At 3:19 P.M. the diameter of the vacuole was only about one-fourth its original length. The bacteria were closely packed around the crimson crystal, forming a dark pink mass closely surrounded by the vacuolar membrane which was still well covered with red granules. A few moments later the vacuole began to increase rapidly in size. At 3:20 P.M. it had nearly reached its original size. During its enlargement the bacteria had become lighter in color, the fluid in the vacuole remained colorless and the neutral-red granules on the surface disappeared. By 3:23 P.M. the bacteria had become colorless, considerably larger and indefinite in outline and the crystal had become nearly colorless. No further changes in color occurred in the content of this vacuole. None of it became yellow. This was doubtless due to excessive dilution of the dye during the enlargement of the vacuole as indicated below. These results were confirmed by observations for shorter periods on many other food-vacuoles in this preparation.

In observations on food-vacuoles in paramecia in other preparations, some of which contained stained yeast-cells, the following was found: Under some conditions the content of the forming vacuole did not rotate, the bacteria were quiet and there was no Brownian movement. This indicates that the fluid in it was very viscous. The newly formed food-vacuoles were usually well covered with neutral-red gran-

ules, but occasionally there were only a few on the surface. Sometimes all the granules left the surface before the vacuole had decreased to minimum in size, but there was no indication that any entered the vacuoles. In preparations which contained but little neutral red, the neutral-red granules did not stain, but the bacteria in the food-vacuoles still became pink after they died. All but a few of the food-vacuoles observed were nearly spherical when they left the pharynx, and all these rapidly became spherical after they had left. The rest were spindle-shaped when they left the pharynx. Most of these rounded up very slowly after they had left and a few of them probably did not become spherical at all. Nearly all the spindle-shaped vacuoles observed were formed in paramecia which had been subjected for twelve hours or longer to relatively strong solutions of neutral red.

In most of the food-vacuoles observed a yellow hyaline layer formed at the surface, as the vacuoles enlarged after having decreased to minimum in size. This layer surrounded a red mass consisting of bacteria and other particles which gradually became uniformly distributed and distinctly yellowish in color. In the rest of the vacuoles observed, no layer formed at the surface, the distribution of the bacteria and other particles keeping step with the enlargement of the vacuoles; but the entire content of these vacuoles eventually became yellow, just as it did in those in which a layer had formed.

Ordinarily the food-vacuoles, after they had enlarged, remained intact until their undigested content was eliminated; but in a few instances two or more were seen to fuse, and some of the vacuoles thus formed were very large.

The results presents show that there is great variation in all the phenomena observed in the food-vacuole, but that the change in the acidity of its content is closely correlated with change in its size. They indicate that, as the vacuole decreases in size, the hydrogen-ion concentration of its content increases in some instances to more than pH 4 and then (as it increases in size) decreases approximately to pH 8. The colors of the neutral-red-containing buffers used in measuring the acidity of the neutral red stained content of the food-vacuoles, were however so indefinitely correlated with the acidity of the buffers that the results obtained are necessarily little more than crude approximations.

#### THE MAXIMUM ACIDITY OF THE CONTENT OF THE FOOD-VACUOLE

*Congo red (pH 3, blue—pH 5, orange)*

As stated above, congo red has been used by several investigators to measure the maximum acidity of the content of the food-vacuole in *Paramecium* and the conclusions reached differ greatly. In attempting to elucidate this diversity I extended the observations which have been made, using *Paramecium caudatum*, *P. nucleatum*, *P. aurelia*, and *P. trichium*.

In specimens of *P. caudatum* mounted in culture fluid containing numerous bacteria and congo red in excess, the following was found: The fluid in the newly formed food-vacuoles was light yellow, the undissolved particles of congo red, yellowish brown and the bacteria, colorless. As the vacuoles decreased in size after they had left the pharynx, the fluid in them decreased greatly in quantity and became light blue, the bacteria died and became dark blue and the particles of congo red became dark blue; then as the vacuoles increased in size, the fluid in them increased greatly



in quantity and became orange, after which the bacteria and the particles of congo red soon also became orange, and remained so until they were eliminated.

In some of these observations the paramecia, after having been a few minutes in the culture fluid containing bacteria and congo red, were transferred with as little fluid as possible, to a smear of polyvinyl alcohol on a slide and covered with a cover-glass. These paramecia became so quiet that they could readily be studied under high magnification. Some of them contained food-vacuoles in which all the changes described above were very distinctly seen. When these food-vacuoles had become minimum in size the color of their content resembled that of buffer pH 3.2. These results therefore indicate that the maximum acidity of the content of the food-vacuoles in *Paramecium* is approximately pH 3.2 and that this is reached when the vacuoles have decreased to minimum in size. These observations were repeated, but yeast-cells which had been stained with congo red were added to the preparations.

The culture from which the paramecia were taken was pH 6.8. They were mounted in this fluid but NaOH was added to some of the preparations, so that a series was obtained which ranged from pH 6.8 to pH 9.8. The stained yeast-cells added were brilliant orange in color, and they remained so in all the preparations. The following results were obtained:

There was no perceptible change in the color in the ingested yeast-cells in any of the preparations until after the food-vacuoles containing them had left the pharynx and had decreased considerably in size. Then, in the preparations, pH 6.8–9, the yeast-cells in vacuoles which contained not more than about six, became sky-blue like buffer pH 3, as the vacuoles decreased in size and then, after the vacuoles had increased in size, brilliant orange again. If the food-vacuoles contained more than about six yeast-cells the extent of change in color from orange toward blue varied inversely with the number of cells in a vacuole. In those which were well filled with yeast-cells and consequently contained but little fluid, there was no appreciable change in color. These vacuoles decreased only slightly in size. The extent of change in color from orange toward blue, i.e., the increase in hydrogen-ion concentration, is therefore correlated with the number of yeast-cells in the vacuoles and the extent of the decrease in their size.

In fluid at pH 9.4 many of the paramecia did not feed, and those which did formed food-vacuoles which were considerably smaller and contained fewer yeast-cells than those formed in ordinary culture fluid. The cells in some of these vacuoles did not appreciably change in color; but those in others became definitely bluish, i.e., about like buffer pH 4. In fluid at pH 9.8 only a few of the paramecia fed and the food-vacuoles formed contained only a few yeast-cells. There was no perceptible change in color in any of these vacuoles, despite the fact that they contained only a few yeast-cells. This seems to show that the extent of change in color from orange toward blue depends upon the alkalinity of the fluid which is ingested with the yeast-cells. The fact, however, that no difference in the extent of change in color was observed in culture fluids which ranged from pH 6.8 to pH 9 indicates that this correlation is not very close.

In some of the observations paramecia were used which had been transferred successively through ten separate portions of fresh sterile culture fluid so as to eliminate nearly all the bacteria. The yeast-cells ingested in this solution changed in color as extensively as those ingested in solutions which contained numerous bacteria. The

increase in the acidity of the fluid in the food-vacuoles is therefore not closely, if at all, correlated with the number of bacteria in it.

There was no perceptible color in the fluid in the food-vacuole in any of the tests made with yeast-cells.

The results obtained with *P. nucleatum* and *P. aurelia* are essentially the same as those obtained with *P. caudatum*, but those obtained with *P. trichium* differed in that no change in color whatever was observed in the ingested yeast-cells. In this species the food-vacuoles usually contain only one yeast-cell when they leave the pharynx (never more than two) and very little fluid. They consequently change but little in size after they have left the pharynx. This is probably why there is no perceptible change in the color of the stained yeast-cells in them.

The results obtained with congo red therefore indicate that the maximum acidity of the content of the food-vacuole in *Paramecium* is approximately pH 3.2. This is in harmony with Bozler's conclusion. Nirenstein and Kalmus contend however, as previously stated, that the maximum acidity is much higher.

Nirenstein (1925) found, just as Bozler had, that ingested congo-red-stained yeast-cells become blue in some food-vacuoles in *Paramecium*, but he contends that Bozler's conclusion is erroneous. He put yeast-cells and coagulated egg albumen which had been stained orange with congo red, respectively into different concentrations of hydrochloric acid and found that, whereas a solution of congo red in N/1000 (pH 3) HCl is blue, neither the yeast-cells nor the egg albumen changed color in concentrations lower than N/30 (pH 1.47) and did not actually become blue until N/10 (pH 1) was reached. He consequently concluded that since stained egg-albumen and yeast-cells became blue in some of the food-vacuoles, the acidity of the fluid in those vacuoles must have been approximately pH 1. Kalmus (1931) repeated Nirenstein's observations and came to the same conclusion.

I added yeast-cells and congo red in excess to a series of Clark buffer solutions (pH 1-9) in test-tubes, heated them to boiling and then left them for 12 hours. At pH 2 and lower, the dye had precipitated, the fluid was very light purplish blue and the yeast-cells were not perceptibly stained. At pH 2.2 a few of the yeast-cells were slightly stained, light purple, the same shade as the fluid. At pH 3 the fluid was densely lavender and about one-half of the yeast-cells were strongly stained and the color was like that of the fluid. At pH 5 the fluid and all the yeast-cells were deep red. At pH 7 the fluid was the same color as at pH 5 but the yeast-cells were light orange. At pH 8 and higher the fluid was red but the yeast-cells were only slightly stained and light yellow in color. Congo red therefore appears to stain yeast-cells most readily in the neighborhood of pH 5.

Some of the red yeast-cells in buffer pH 5 were transferred to a series of buffers (pH 1-3) containing congo red and examined from time to time for two hours. At pH 1 and 1.2 the yeast-cells became densely sky-blue, at pH 1.4 purplish blue, at pH 2 and 3 definitely purple.<sup>7</sup>

Some of the red yeast-cells from the pH 5 buffer were added to culture fluid containing numerous paramecia. The yeast-cells were freely ingested. No change in color was observed in any of the food-vacuoles which were well filled with yeast-

<sup>7</sup> It is not clear why yeast-cells do not stain at all with congo red at pH 1 and 1.2 but become densely blue at these hydrogen-ion concentrations if they are first stained at higher concentrations.

cells, but in some of those which contained only a few, they unquestionably became as blue as those in buffers pH 1 and 1.2 and then, as the vacuoles increased in size, red again. In others they became purple of various shades so that all colors obtained in buffers pH 1-3 could be matched.

These results appear therefore to support Nirenstein's conclusion that the maximum acidity of the content of some of the food-vacuoles in *Paramecium* is approximately pH 1. The salt, protein and other errors for congo red are however so great that Clark (1928) and others maintain that the results obtained with it can be considered only as crude approximations. Moreover, in measurements made with a glass electrode, I found that congo red decreases the acidity of solutions and that in saturated solutions of congo red this decrease is great, i.e., from pH 3.06 to pH 6.61 in N/1000 HCl. Nirenstein's conclusion regarding the maximum acidity of the content of the food-vacuole, based on the assumption that congo red does not affect the acidity of solutions of hydrochloric acid, is consequently equivocal. I therefore repeated the experiments on congo red described above, with thymol blue and meta cresol purple, both of which have only very small errors and only slightly affect the acidity of solution to which they are added.

*Thymol blue* (pH 1.2, red—pH 2.7, yellow) and *meta cresol purple* (pH 1.2, red—pH 2.8, yellow)

In buffer solutions containing thymol blue, yeast-cells stain most readily at pH 3-5 and become lemon yellow. At pH 1-1.6 they do not stain perceptibly, but if yellow yeast-cells are put into these buffer solutions they become definitely pink of the same shade as the solutions.

*Paramecia* ingest the stained yeast-cells freely except in preparations in which the dye is too concentrated or the fluid too strongly acid. In every preparation studied the density of the yellow color of the yeast-cells in the forming food-vacuoles increased considerably. This is doubtless due to increase in the concentration of the dye, owing to impermeability of the vacuolar membrane to it and loss of water. After the food-vacuoles had left the pharynx, the yeast-cells in most of the vacuoles remained yellow but in some in nearly every preparation they unquestionably changed color and became as pink as those in buffer pH 1.4, and then as the vacuoles increased in size, yellow again.

The results obtained with meta cresol purple are essentially the same as those obtained with thymol blue. The ingested, stained yeast-cells remained yellow in most of the food-vacuoles, but in some they changed color as the vacuoles decreased in size and clearly became as pink as those in the buffer pH 1.4, and then yellow again as the vacuoles enlarged.

The results presented seem to demonstrate that while the maximum acidity of the content of the food-vacuoles in *Paramecium* differs enormously, it is at least as high as pH 1.4 in some of them. If this is true it is phenomenal, for culture fluid very much lower (even as low as pH 3.5) is instantly fatal to *paramecia*. The relatively high concentration of acid in the food-vacuole without injury to the adjoining cytoplasm is convincing evidence in support of the contention presented above that the vacuolar membrane is impermeable to acid.

## THE MAXIMUM ALKALINITY OF THE CONTENT OF THE FOOD-VACUOLE

Four dyes were used in the observations on the maximum alkalinity of the content of the food-vacuoles in *Paramecium*, nile blue (Grubler, 1915, pH 7, blue—pH 8, purple); phenol red (pH 6.8, yellow—pH 7.4, pink); brom thymol blue (pH 6, yellow—pH 7.6, blue), and cresol red (pH 7.2, yellow—pH 8.8, red). The observations made with congo red were repeated with each of these four dyes except that only two species (*P. caudatum* and *P. nucleatum*) were used and that the hydrogen-ion concentration of the culture fluid in which the paramecia were mounted, extended over a wider range. No difference was observed in the results obtained with the two species.

*Nile blue*

In the paramecia mounted in culture fluid containing nile blue, numerous food-vacuoles with many bacteria were formed in all the preparations except those which were so strongly alkaline that they were definitely toxic. These preparations were examined under low and high magnification from time to time for six hours. The content of the food-vacuoles was practically colorless until after the vacuoles had decreased considerably in size; then the bacteria in them became deep sky-blue in all the preparations and appeared to be dead, but the fluid remained colorless. Later the vacuoles increased in size, but there was no appreciable change in the color of either the fluid or the bacteria in them. There was no indication of a change to a purplish tint in the content of any of them. The buffer solutions containing Grubler's nile blue were sky-blue at pH 6.8, slightly purplish at pH 7.2, and distinctly purple at pH 7.6 and higher. The results presented, therefore, seem to show that the content of the food-vacuoles certainly did not become as alkaline as pH 7.6 in any of them, and probably not as alkaline as pH 7.2.

Yeast-cells stained with nile blue were mounted respectively in acid (pH 6.8) and alkaline (pH 8.6) culture fluids containing paramecia. The yeast-cells were freely ingested by the paramecia in all the preparations studied. In the acid culture fluid they were sky-blue and no perceptible change in color occurred as they passed through the body. In the alkaline culture fluid they were purple and they usually remained purple, but occasionally, if the food-vacuoles contained only one or two, they became blue as the vacuoles decreased in size and then purple again after they had enlarged.

Some of the blue yeast-cells were mounted in each of the buffer solutions in the series prepared. They remained blue in all below pH 7.6 and became only slightly purplish at pH 7.8. The results presented above indicate, therefore, that the content of some of the food-vacuoles formed in alkaline culture fluid became at least as alkaline as pH 7.8; but the fact that the yeast-cells became purple in only a very small percentage of the food-vacuoles shows that it rarely becomes as alkaline as this. However, the fact that the yeast-cells did not become purple in any of the vacuoles formed in acid culture fluid indicates that the maximum alkalinity of the content of the food-vacuoles is correlated with the hydrogen-ion concentration of the surrounding medium.

*Phenol red*

Paramecia can withstand a surprisingly strong solution of phenol red. If the solution is alkaline the cytoplasm becomes distinctly yellowish green. The bacteria in the culture fluid used containing phenol red were colorless and the fluid was yellow or pink, depending upon the hydrogen-ion concentration. In the food-vacuoles the bacteria, regardless of the hydrogen-ion concentration of the culture fluid, became dark yellow as the vacuoles decreased in size, but the fluid around them remained colorless. Then as the vacuoles enlarged the fluid and the bacteria became faintly but distinctly pink (pH 7.4) in some of them and colorless in the rest. These results are essentially in accord with those obtained by Shipley and DeGaris (1925).

Stained yeast-cells became distinctly yellow in the acid and distinctly pink in the alkaline culture fluids used. In the food-vacuoles the pink yeast-cells became yellow and the yellow ones remained yellow as the vacuoles decreased in size, the fluid remained colorless. In about five percent of the vacuoles, especially those which contained only a few yeast-cells, both kinds became faintly but distinctly pink in color similar to stained yeast-cells in buffer pH 7.4, as the vacuoles enlarged. In the rest they remained either yellow or became colorless. No definite correlation between the hydrogen-ion concentration of the surrounding medium and the changes in the color of the content of the vacuoles was observed.

These results indicate that the maximum alkalinity reached in the food-vacuoles varies greatly in different vacuoles but that it never exceeds pH 7.4. Phenol red is, however, not very satisfactory for the measurement of the hydrogen-ion concentration of the content of food-vacuoles; for the changes in color are rather indefinite and the color of buffer solutions is at best not closely correlated with their hydrogen-ion concentration.

*Brom thymol blue*

The bacteria in the culture fluid, both acid and alkaline, containing brom thymol blue were colorless. They were consequently colorless when they entered the food-vacuoles. No changes in color in them or in the fluid around them was observed as they passed through the body.

In the alkaline culture fluid used the stained yeast-cells became blue, and in the acid culture fluid yellow. The blue yeast-cells which were ingested became yellow almost immediately after the food-vacuoles left the pharynx, and in all the food-vacuoles which contained more than about four, they remained yellow until they were eliminated or became colorless; but in the food-vacuoles which contained fewer than about four they became (after the vacuoles had enlarged) greenish blue, about the shade assumed by stained yeast-cells in buffer pH 7 or 7.2. No difference in shade was observed in the acid and the alkaline culture fluids used.

These results indicate, therefore, that the maximum alkalinity attained by the content of the food-vacuoles is not greater than pH 7 or possibly 7.2, i.e., not so great as the results obtained with Nile blue and phenol red indicate. The changes in the color of yeast-cells stained with brom thymol blue is, however, so elusive in the food-vacuoles that they indicate but little concerning the hydrogen-ion concentration of the fluid around them.

*Cresol red*

The bacteria in culture fluid containing cresol red were colorless when they were ingested, and no change in color was observed in them as they passed through the body.

The stained yeast-cells became light yellow in the acid culture fluid used and light pink in the alkaline. In the food-vacuoles they remained or became yellow as the vacuoles decreased in size and then, with a very few exceptions, either became colorless or remained yellow until they were eliminated, i.e., they did not change color after the vacuole had enlarged. In a few instances, however, the yellow yeast-cells assumed a light pink color, about like buffer pH 7.8.

It is evident that the results obtained with the four dyes used differ considerably. They seem to indicate however that the alkalinity of the content of some of the food-vacuoles is nearly, if not quite so high as pH 7.8 but that it is much lower in most of the vacuoles. They also indicate the maximum alkalinity varies somewhat with the acidity of the fluid ingested.

## THE ORIGIN OF THE ACID AND THE BASE IN THE FOOD-VACUOLE

It is widely held that the acid and the base in the food-vacuole are secreted by the adjoining cytoplasm (Greenwood and Saunders, 1894; Nirenstein, 1905; Lund, 1914; Howland, 1928; Claff et al., 1941; and others). However, Mast (1942) concludes that in *Amoeba* the acid originates in the vacuoles and increases in concentration owing to impermeability of the vacuolar membrane to the acid and loss of water, and that the subsequent increase in alkalinity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles, that is, that "the cytoplasm secretes neither acid nor base." And Mast and Bowen (1944) conclude that in the Peritricha, "The increase in the acidity of the content of the vacuole is probably due to the production of acid, owing to metabolism in the peristome, the vestibulum and the pharynx and impermeability of the vacuolar membrane to organic acid, resulting in its retention and consequent concentration as the vacuole decreases in size." Mast and Bowen (p. 213) present strong evidence in support of their conclusion. This evidence applies equally well to the food-vacuoles in *Paramecium* and leads to the same conclusion. It should be emphasized, however, that secretion of acid by the cytoplasm adjoining the vestibulum and the pharynx is by no means ruled out.

The increase in alkalinity in the food-vacuole in *Paramecium* is doubtless brought about in the same way as it is in the Peritricha, i.e., by entrance of alkaline fluid from the cytoplasm. According to these views the cytoplasm adjoining the food-vacuoles does not secrete either acid or base.

## THE FUNCTION OF THE CHANGES IN THE HYDROGEN-ION CONCENTRATION IN THE FOOD-VACUOLE

It is maintained by Hemmeter (1896), Nirenstein (1925), Howland (1928), and Claff et al. (1941) that the organisms ingested by protozoa are killed by the acid in the food-vacuoles and that this is its primary if not its only function. There is, however, strong opposition to these contentions.

Fortner (1933) holds that in *Paramecium caudatum* the ingested organisms are killed by a toxic substance. He asserts that this substance is formed in the neutral-

red granules, and that these granules pass through the vacuolar membrane into the food-vacuole shortly after it has left the pharynx, and release the substance there.

These assertions are certainly not strictly valid, for as demonstrated above, the neutral-red granules do not enter the food-vacuole. It might be, however, that since some of them are in close contact with the vacuolar membrane when the food-vacuole leaves the pharynx, toxic substance diffuses from them into the vacuole and kills the ingested organisms. It is however far more likely that if toxic substance is involved in this phenomenon, it is secreted by the wall of the pharynx and taken up by the fluid which passes into the forming food-vacuoles, and that it there (owing to impermeability of the vacuolar membrane and loss of water) increases in concentration and consequently becomes lethal. At any rate, there is much evidence that the pharynx secretes substances which pass into the forming food-vacuole.

Fortner (1933) found that if numerous crushed bacteria are added to culture fluid containing live bacteria and paramecia the ingested bacteria are not killed, and that the vacuoles, as previously stated, do not decrease in size. This seems to show that death of the bacteria depends upon decrease in the size of the vacuoles. If this is true, it lends support to the hypothesis that the bacteria in the food-vacuole are killed by toxic substance which has been concentrated in the food-vacuoles owing to loss of water and impermeability of the vacuolar membrane to the toxic substance.

Mast (1942) in observations on *Amoeba* and Mast and Bowen (1944) in observations on the Peritricha present strong evidence that the acid in the food-vacuoles in these protozoa does not become sufficiently concentrated to kill the ingested organisms and the results presented above seem to show that this also obtains for most of the food-vacuoles in *Paramecium*. Mast concludes that in *Amoeba* the primary lethal factor in the food-vacuole is reduction of oxygen, owing to respiration and loss of water, and Mast and Bowen accept this view in reference to the Peritricha, but they present no evidence in support of it and none was obtained in the present observations on *Paramecium*.

No one has observed any indication of digestion in the food-vacuole during its acid phase. Nirenstein (1905) thinks, however, that there are invisible preliminary changes. However this may be, it is practically certain that the osmotic concentration of the fluid in the vacuole increases greatly during this phase, and it may well be that this is caused by hydrolysis of complex compounds, owing to the presence of acid, and that this causes the rapid inflow of fluid from the cytoplasm, which probably contains digestive enzymes. If this is true, the acid in the food-vacuoles obviously functions, at least indirectly, in digestion. There are consequently several hypotheses concerning the function of the acid in the food-vacuole but there is not much evidence in support of any of them.

It has been conclusively demonstrated that digestion in the protozoa takes place largely, if not entirely, in the food-vacuoles during the alkaline phase, but since this phase seems to be merely the result of inflow of fluid from the cytoplasm, digestion is not correlated with anything in the nature of secretion of alkaline substance.

## DIGESTION

Nirenstein (1905) made detailed observations on bacteria, yolk granules, fat globules, and starch grains in the food-vacuoles in *Paramecium caudatum*. He says he observed no indication of digestion in any of these substances until after the fluid

in the vacuoles had become alkaline, but that after this had occurred the bacteria and the yolk granules gradually decreased in number and finally disappeared entirely and some of the starch grains corroded. He was at this time in doubt about the fate of the fat globules, but he later (1910) presented evidence which strongly indicates that fat is hydrolyzed in the food-vacuoles.

Nirenstein's views concerning the time of digestion in the food-vacuoles in *Paramecium* and the digestion of proteins have been adequately confirmed; his contention that paramecia digest starch is supported by results obtained by Pringsheim (1928) and Zingher (1933) in observation on *Paramecium caudatum*; and his contention that they digest fat is indirectly supported by results obtained by Dawson and Belkin (1929), Mast (1938), and Wilber (1942) in observations on *Amoeba* and *Pelomyxa*.

I repeated Nirenstein's observations and obtained results which are in harmony with his. It should be emphasized, however, that in my experiments only a very small proportion of the starch (wheat and potato) ingested by the paramecia was digested. Observations by Greenwood (1886), Meissner (1888), and Mast and Hahnert (1935) indicate that this also obtains for *Amoeba*. Indeed, it is doubtful whether in this organism any starch is digested except that which is in other organisms which have been ingested.

#### THE ORIGIN OF THE DIGESTIVE ENZYMES AND THE FUNCTION OF THE NEUTRAL-RED GRANULES AND THE MITOCHONDRIA

The fact that protein, starch and fat are digested in the food-vacuoles in *Paramecium* shows that these vacuoles contain peptidase, amylase, and lipase, and the fact that digestion does not begin until after the fluid in the vacuole has become alkaline shows that these enzymes are active in a basic medium. The questions now arise as to where these enzymes originate and how they get into the food-vacuoles.

Prowazek (1898) who, in observations on paramecia, discovered the neutral-red granules and their tendency to aggregate at the surface of the forming food-vacuole, maintains that they contain digestive enzymes. He consequently called them "Fermentträger". He offers no explanation as to how the postulated enzymes in the granules get into the vacuole, but Nirenstein (1905) who accepts Prowazek's contentions, holds that the granules pass through the vacuolar membrane and carry the enzymes into the vacuole. He asserts that, in paramecia in culture fluid containing neutral red, he observed red granules appear in food-vacuoles shortly after they had left the pharynx. He consequently concluded that the red granules in the food-vacuoles came from the cytoplasm and passed through the vacuolar membrane into the food-vacuoles and that the enzymes in the vacuoles are carried in by these granules.

These conclusions are supported by Rees (1922), Bozler (1924), Fortner (1926), Volkonsky (1929), Muller (1932), and MacLennan (1941, p. 129). All these investigators hold that in *Paramecium* the neutral-red granules actually pass through the vacuolar membrane and carry enzymes into the food-vacuoles. None of them, however, maintains that he saw this. Their contention is based largely on Nirenstein's observation of the appearance of red granules in the food-vacuoles. It is, however, obvious that these granules may have entered the food-vacuoles by way of the pharynx. The evidence in support of the contention that neutral-red



granules pass from the cytoplasm into the food-vacuoles in *Paramecium* is therefore very weak. There is, however, evidence which strongly indicates that in some other organisms neutral-red staining substance actually passes from the cytoplasm into the food-vacuoles.

MacLennan (1936) asserts that in *Ichthyophthirius* neutral-red staining substance becomes attached to particles of food which are naked in the cytoplasm, that these particles aggregate, and that a membrane then forms, enclosing them, together with some fluid, in a vacuole. He thinks that the neutral-red staining substance contains digestive enzymes. Hopkins and Warner (1946) hold similar views regarding the food-vacuoles in *Entamoeba histolytica* and they maintain, moreover, that they actually saw globules pass through the vacuolar membrane into and out of the food-vacuoles.

The idea that granules or globules carry enzymes into the food-vacuoles, is consequently widely held. There is, however, evidence which very strongly indicates that this does not obtain in *Paramecium*.

Khainsky (1911) studied the food-vacuole of *Paramecium caudatum* in fixed and stained specimens and in living specimens. He obtained no evidence which indicates that granules or globules pass from the cytoplasm into the food-vacuoles. He maintains, however, that neutral-red staining globules form in the vacuole and pass out through the vacuolar membrane. Koehring (1930) made extensive observations on neutral-red granules in various protozoa. She concludes that they function as enzyme carriers in all, but that they do not enter the food-vacuoles. Referring to *Paramecium caudatum* she says (p. 67): "As the new vacuole is being formed they [the neutral-red granules] gather at the membrane, bombarding it like hailstones, but making no impression on the firm surface. Then as this vacuole flows away attached by its canal . . . some of the granules leave this vacuole and return to the next, which is already in the process of formation. Those left continue bombardment of the vacuole as the pink color slowly forms within."

Dunihue (1931) asserts that in *Paramecium caudatum* the surface of the forming food-vacuoles becomes "closely packed" with neutral-red staining globules "which remain firmly attached throughout most of the digestive period" and do not enter the vacuoles.

As stated in a preceding section, I made under most favorable conditions intensive observations on the neutral-red granules at the surface of food-vacuoles throughout their entire existence and obtained no evidence indicating that visible granules or globules of any kind ever pass through the vacuolar membrane into or out of the food-vacuoles in *Paramecium*. What, then, was the origin of the red granules observed by Nirenstein in the food-vacuoles in *Paramecium*?

These granules doubtless originated in substance ingested by the paramecia. At any rate it has been clearly demonstrated that this occurs in *Amoeba proteus* (Mast and Hahnert, 1935; Mast and Doyle, 1935). It can therefore be concluded that the neutral-red granules found in the cytoplasm in *Paramecium* do not enter the food-vacuoles. This does not, however, demonstrate that they are not involved in digestion. If they contain enzymes it may be that in aggregating at the surface of the food-vacuoles the concentration of enzymes is increased and that this facilitates their diffusion into the vacuoles. The facts, however, that there is a marked flow of fluid out of the vacuole during the entire time that the aggregation of these granules is prominent and that most of the granules usually leave the surface of the vacuoles

long before digestion begins, seem to militate against this possibility. Moreover, Hall and Dunihue (1931) and Mast and Bowen (1944) found that in *Vorticella* and other Peritricha the neutral-red granules do not aggregate at the surface of the food-vacuoles, and Mast (1926) found that in *Amoeba* there are none. They are therefore certainly not functional in digestion in the latter and very probably not in the former. The evidence in support of the contention that they function in digestion in *Paramecium* is therefore negligible. What, then, is their function in this organism?

Fortner (1926, 1928) maintains that they originate in the cytoplasm adjoining the nucleus, that they contain protein and fat, and that they decrease greatly in number during starvation. If these contentions are valid, the composition of the granules and their disappearance seem to suggest that they are reserve food; but, if this is what they are, why do they originate near the nucleus, and why do they aggregate at the surface of the food-vacuole during its formation? The function of the neutral-red granules in *Paramecium* is apparently still decidedly hazy.

Horning (1926a) contends that in *Amoeba* sp. janus-green staining bodies (mitochondria) aggregate among particles of food in the cytoplasm and that a membrane then forms and encloses the food and the mitochondria in a vacuole. He says that in *Paramecium* sp. mitochondria "are extruded from the cell protoplasm [through the vacuolar membrane] into the food vacuoles" during the alkaline phase. He concludes that the mitochondria in these protozoa function in carrying digestive enzymes to the ingested food. Volkonsky (1934) does not agree with Horning. He maintains that the mitochondria do not enter the food-vacuoles and that they do not take part in digestion in protozoa.

As stated above, I was unable to see any particles pass from the cytoplasm into the food-vacuoles in *Paramecium*. Hall and Nigrelli (1930) could not obtain any evidence of mitochondria in the food-vacuoles in *Vorticella*, and Mast and Doyle (1935) in an intensive study of the movement of the mitochondria in *Amoeba proteus* under very favorable conditions concluded that they do not pass into the food-vacuoles. They found that the mitochondria move about in the cytoplasm freely and often come in contact with the surface of the food-vacuoles in all stages of development and at times form aggregations at the surface, but that none pass into the vacuoles. These observations and others led them to conclude that the mitochondria in *Amoeba* "probably function in transferring substances from place to place in the cytoplasm."

Holter and Kopac (1937) maintain that in centrifuged specimens of *Amoeba proteus*, the mitochondria are in a fairly well defined stratum but that the enzyme, peptidase, is not stratified. They consequently conclude that this enzyme is not associated with mitochondria. However, Holter and Doyle (1938) in similar tests found that amylase and mitochondria in *Amoeba* tend to accumulate in the same stratum. These results seem to indicate that this enzyme is attached to the mitochondria. The authors do not, however, hold that this association is proved.

The evidence in support of the contention that the mitochondria and the neutral-red granules in *Paramecium* are enzyme carriers is therefore, as yet, far from convincing. Where, then, do the digestive enzymes in the food-vacuoles come from?

Some doubtless come from the organisms and other substances which have been ingested and the rest are, I think, as previously stated, carried from the cytoplasm into the vacuoles with the fluid that enters during the rapid enlargement of the vacuoles at the beginning of the alkaline phase. Where and how the enzymes in the

cytoplasm originate is not known. Fortner (1926), as stated above, maintains that the neutral-red granules are formed in the cytoplasm adjoining the nucleus. He holds that the enzymes are closely associated with these granules and consequently concludes that they also originate in this region. The validity of the contention that the enzymes are closely associated with the neutral-red granules is, however, very doubtful. The evidence in support of Fortner's conclusion is, therefore, very weak. It is known however that in *Chilomonas paramecium* enzymes are synthesized in the protoplasm, for this organism can be grown on known simple chemical compounds (Mast and Pace, 1933), but how and where they are synthesized and what they are, chemically, is not known, and this can also be said of the enzymes in other protozoa.

### SUMMARY

1. The feeding apparatus in *Paramecium* consists of a shallow ciliated groove, a ciliated tube which leads into the body, and a bundle of fibers (esophageal fibers) which extend from the tube nearly to the posterior end of the body. The tube is composed of an outer part (the vestibulum) and an inner part (the pharynx).

2. Paramecia ingest all sorts of small particles, but more digestible than indigestible ones. Selection takes place in the vestibulum and the proximal end of the pharynx.

3. In forming a food-vacuole, the cilia in the pharynx force fluid with particles in suspension against the membrane over the distal opening of the pharynx, producing a sac, the esophageal sac.

4. As the esophageal sac enlarges, the particles in suspension in it become greatly concentrated, owing largely, if not entirely, to the passage of water out through the membrane into the cytoplasm.

5. A portion of this sac is constricted off, as a food-vacuole, probably by the action of the esophageal fibers.

6. The initiation of the constriction of the sac is probably due to periodicity in the constrictive action of the fibers, the size of the sac, and the composition of its contents.

7. There is much variability in the size of the food-vacuoles. This is correlated with the quantity and the quality of the particles in the surrounding fluid, the chemical composition of this fluid, the rate of ingestion, the rate of loss of water from the esophageal sac, and the length of the intervals between consecutive constrictions of the esophageal fibers. The frequency of formation of food-vacuoles is correlated with the quantity and the quality of the particles in the surrounding fluid and the acidity, and the temperature of this fluid. The shape of the food-vacuoles depends largely, if not entirely, upon the viscosity of their content.

8. After the food-vacuole has left the pharynx it passes rapidly on a fixed course toward the posterior end of the body, and slowly on a varied course to the anus. The former is probably due to the action of the esophageal fibers; the latter is due to cyclosis.

9. On its course through the body, the food-vacuole usually decreases greatly in size, and the acidity of its content increases greatly; then it enlarges very rapidly and the acidity of its content decreases greatly. The extent of these changes varies enormously. Under some conditions there are no perceptible changes; under others

the acidity in some vacuoles increases to a maximum at least as high as pH 1.4 and then decreases approximately to pH 7.8.

10. There is no "preliminary alkaline phase" of the food-vacuole.

11. The change in acidity is definitely correlated with change in size. The changes in size are due to difference between internal and external osmotic concentration and the action of the stretched vacuolar membrane. The increase in acidity is probably due to secretion of acid by the cytoplasm adjoining the vestibulum and the pharynx and to impermeability of the vacuolar membrane to hydrogen-ions, and loss of water. The decrease in acidity is due to entrance of alkaline fluid from the cytoplasm.

12. The increase in acidity probably causes hydrolysis and thereby increase in osmotic concentration resulting in inflow of fluid containing digestive enzymes.

13. Death of ingested living organisms is probably largely due to toxic substance produced by the pharynx and concentrated in the food-vacuole, owing to impermeability of the vacuolar membrane to it, and loss of water.

14. Paramecia digest protein, fat, and starch. Digestion takes place during the alkaline phase of the food-vacuole. The enzymes involved originate in the cytoplasm and are carried into the food-vacuole by the cytoplasmic fluid which enters during its rapid enlargement.

15. The neutral-red granules and the mitochondria are probably not involved in digestion.

16. All these phenomena are essentially the same in the four species studied, namely *P. caudatum*, *P. nucleatum*, *P. aurelia*, and *P. trichium*.

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# POISONING AND RECOVERY IN BARNACLES AND MUSSELS<sup>1</sup>

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## INTRODUCTION

Previous investigations have shown that the prevention of the attachment of organisms to ships' hulls by antifouling paint is related to the rate of solution of the toxic material, and hence, to its concentration in the sea water at the paint's surface (Ketchum, Ferry, Redfield, Burns, 1945). The present investigation was undertaken to test the effectiveness of different metallic salts as poisons for two important types of fouling organisms and to evaluate them under conditions in which the concentration and ionic form of the toxic materials were accurately known. In earlier attempts to test lethal action, supposedly toxic materials have been incorporated in paints applied to test-panels or to the inside surfaces of containers and the degree of fouling resistance noted, but in most cases no adequate measure was made of the concentrations of the toxics which were actually emitted by the paint. In other tests, fouling organisms were placed in containers of sea water to which known amounts of toxics had been added but without adequate knowledge of the ionic transformation of the toxics which ensue in the presence of sea water (Bray, 1919; Edmondson and Ingram, 1939; Orton, 1929-30; and Parker, 1924).

We proposed, therefore, to conduct direct tests of the action of various individual metals in graded concentrations and in different chemical forms, on two important types of fouling organisms; namely, barnacles and mussels. We desired also to investigate the relationship between the concentration of various toxics and the exposure time necessary to kill, in order to ascertain the most efficient concentration of the poison for the destruction of fouling organisms.

A second purpose was to follow the course of poisoning and recovery in barnacles and mussels when copper was used as the poison. It was desired to measure the amount of copper in the tissues and to discover whether death from copper poisoning was brought about specifically by the accumulation of a certain amount of the poison in the tissues or by the entrance of the copper into the body at a certain rate or for a critical period of time. Finally, it was desired to ascertain from how great a dose of copper an animal could recover, and to learn whether, after recovery, the animal was more or less susceptible to subsequent exposures of the toxic.

I am indebted to Mrs. Dayton Carritt for assistance in carrying out the experiments, to Mrs. Barbara Mott Ferry for chemical analyses, to Dr. John Ferry and Dr. Gordon Riley for furnishing toxics, and to Mr. Porter Smith for technical assistance. I am indebted to the University of Miami for the use of facilities at the

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Marine Laboratory. I also wish to thank Dr. Walton Smith and Dr. Charles Pomerat for valuable cooperation.

#### OBSERVATIONS ON ADULT BARNACLES AND MUSSELS

The effectiveness of various poisons was investigated first using the barnacle as a test animal, since the barnacle is generally the most serious offender as a fouling organism. Adult barnacles were employed for the majority of the experiments at Woods Hole, but one set of tests was conducted on nauplii which had been discharged in a laboratory tank. Although attachment of the barnacle takes place previous to the adult stage, there is evidence that a poisoned barnacle is less securely attached than a normal animal and may be more easily dislodged. Special interest centers about the poisoning of the cyprid, since this is the attaching stage. In view of the fact that cyprids could not be obtained in sufficient numbers at Woods Hole, experiments on this stage were conducted at Miami Beach, Florida. Mussels, which are of relatively large size, were found advantageous for other tests, especially those involving the copper assay of living tissue.

#### *Source and preparation of material*

Certain of the present tests were carried out with *Balanus balanoides* growing on stones obtained near low tide mark at Woods Hole. In later tests *Balanus eburneus* was employed, obtained through the kindness of Dr. Stanley Cobb, from a wharf in Cotuit Harbor. Parts of the wharf were removed and transported to the laboratory tanks. The barnacles were kindly identified by Dr. Richard McLean.

For the experiments with *B. balanoides* the material was collected on the same day that the tests were begun. Single stones bearing 10 to 30 barnacles (ranging from 3 mm. to 10 mm. in diameter) were placed in individual beakers or culture dishes containing from 250 cc. to 500 cc. of sea water with or without toxic. For the work using *B. eburneus*, a stock of animals attached to fragments of wood was maintained in large battery jars provided with running sea water. In preparation for the tests it was necessary to split and saw the pieces of wood into blocks of convenient dimensions. Blocks about 4 cm. to 6 cm. square, each bearing 10 to 20 barnacles ranging from 4 mm. to 20 mm. in diameter, were similarly placed in individual containers. In other experiments larger blocks bearing several hundred barnacles were placed in battery jars of 8 liters capacity or more.

With both species of barnacles it was found that when the sea water was renewed daily, the animals in the individual containers remained in a healthy condition for several weeks. There were evidently enough food particles in the sea water supplied to provide a maintenance diet. Faecal material was produced in abundance but the barnacles grew little, if at all.

The mussels employed were the common species in the Woods Hole region, *Mytilus edulis*, obtained from the dock and float of the Oceanographic Institution. The mussels were placed individually or in batches in culture dishes or large battery jars. The animals began attaching their byssus threads to the bottoms or sides of the containers almost immediately. If the sea water was renewed each day, the mussels continued in healthy condition indefinitely, apparently obtaining sufficient nutriment from the food particles present in the sea water.

*Behavior during poisoning*

When barnacles were placed in the toxic media, they opened their shells and began raking or filtering the water with their cirri as soon as did the controls in fresh sea water. Ordinarily this took place within a few minutes. If the concentration of the poison was such as to kill the barnacle within two or three days, the animals would begin to show signs of bad condition after about 12 hours. The first symptom was the slowing down of the beat of the cirri and soon thereafter their sweep became reduced in extent. As poisoning progressed, the movement of the cirri became still slower and more curtailed until they opened to only one-half or one-quarter of their usual scope. When the poisoned barnacle stopped filtering, it tended to come to rest with the cirri half extended, in contrast to the healthy animal in which the cirri are drawn completely in and the shell tightly closed during rest. In the poisoned animal, the shell could at first be caused to close by prodding it with a needle. After further poisoning, when no response could be elicited by stimulation with the needle, the barnacle was considered dead. The following symbols are used in the tables to designate these conditions:

++++	normal rapid sweep of cirri
+++	full sweep but slow
++	incomplete sweep
+	open, inactive, but reacts to touch
0	dead

If the barnacle was replaced in fresh sea water (preferably running sea water) within a day of the time it reached the next to last condition (+), it would usually recover completely its full activity after a few days.

In the case of the mussel, the change in condition as poisoning progressed was less easily observed. In the higher concentrations of the toxics, the animals failed to attach themselves to the walls of the containers and tended to remain closed for long periods. When placed in weaker solutions, however, many of the mussels became strongly attached and remained with their shells open to the normal extent to allow the inflow and outflow of water. In either case, after poisoning had progressed to a certain point, the valves were found gaping wide open. When this condition was first reached, the animal responded to prodding by closing slowly. A day later, or less, no response would follow such stimulation and the animal was considered dead. Once the mussel reached the stage in which it gaped open more than normal, though it might still react, it would not recover when placed in fresh sea water. In fact, mussels were frequently observed to die many days after having been transferred from the toxic solution into fresh sea water although outwardly they appeared to be in normal condition in the interim.

*Toxicity of various metallic salts to barnacles*

The toxicity of the following metals was investigated: copper, mercury, silver, and zinc. The last three of these were tested as the ions which resulted from the introduction into the sea water of  $\text{HgCl}_2$ ,  $\text{AgSO}_4$ , and  $\text{Zn}(\text{NO}_3)_2$  respectively. The copper, on the other hand, was tested not only in the form of basic cupric carbonate, but also as cupric citrate, cupric tartrate, cupric salicylate, and cupric para amino-

benzoate. The interest in these other compounds of copper lies partly in the possibility of revealing important differences in toxicity. Of further significance is the fact that these substances are much more soluble in sea water than is basic cupric carbonate. Therefore, when copper is supplied in the form of these more soluble ions, it is possible to establish a high concentration of the toxic in the water adjacent to the surface from which the material is free to dissolve.

Individuals of *Balanus balanoides* were placed in beakers or jars containing graded concentrations of toxics in sea water with a control for each series. Since the media were renewed each day, the danger of significant changes in concentration due to such causes as adsorption was minimized. The minimum concentrations necessary to kill 90 per cent of the barnacles after continuous exposures of two days and five days are indicated in Table I. Among the various forms of copper, the

TABLE I

*The concentrations of various metallic salts necessary to kill 90 per cent of adult barnacles after continuous exposures for the indicated periods. Concentrations are expressed as milligrams of metal per liter of sea water*

	Toxic	Concentration lethal in 2 days	Concentration lethal in 5 days
<i>Series I</i>		mg./liter	mg./liter
<i>Balanus balanoides</i> from Woods Hole harbor	Basic Cu Carbonate	0.35	—
	Basic Cu Carbonate	0.48	—
	Cu Citrate	0.60	0.18
	Cu Citrate	0.60	0.30
	Cu Tartrate	0.58	0.17
	HgCl <sub>2</sub>	1.0	0.5
	Ag <sub>2</sub> SO <sub>4</sub>	0.4	0.2
	Zn(NO <sub>3</sub> ) <sub>2</sub>	32.	8.0
<i>Series II</i>			
<i>Balanus eburneus</i> from Cotuit harbor	Basic Cu Carbonate	0.28	0.14
	Cu citrate	0.55	0.14
	Cu salicylate	0.90	0.45
	Cu para aminobenzoate	—	0.50

basic cupric carbonate was the most effective but the concentrations of the citrate and tartrate necessary to kill were only slightly greater. The toxicity of silver as silver sulphate was of the same order of magnitude but somewhat greater concentrations of mercury as mercuric chloride were required. The effectiveness of zinc, on the other hand, was very much less than any of the other substances tested (cf., Riley, 1943).

Experiments with *Balanus eburneus* indicated similarly that the lethal action of cupric citrate is somewhat less than that of basic cupric carbonate although the magnitude of the difference is not great. The toxicity of cupric salicylate and cupric aminobenzoate were somewhat less than the citrate.

In view of the fact that the toxicity of cupric citrate was found to be almost as great as that of basic cupric carbonate, and since stock solutions of higher and less

variable concentrations could be maintained, the tests which follow were conducted with the citrate.

*Relation between concentration of cupric citrate and killing time*

**Barnacles**

With the higher concentrations of cupric citrate in which killing occurred within a few days, adult barnacles all succumbed at about the same time, but at the lower concentration it was difficult to determine accurately when the barnacles should be considered dead. At still greater dilutions of the toxic, it was found that most of the animals lived on in a feeble condition for two or three weeks or more. Furthermore, there was considerable variation from experiment to experiment, which was no doubt due not only to individual differences in the batches of barnacles, but also

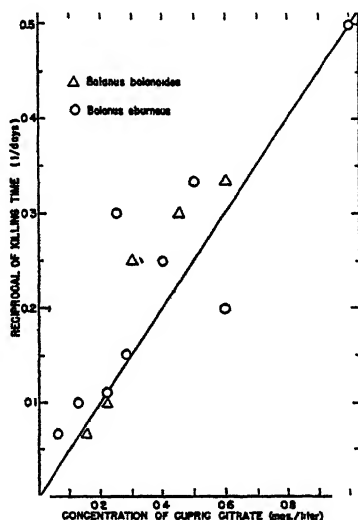


FIGURE 1. The relation between killing time and concentration of toxic for two species of barnacles.

to unavoidable changes in the seasonal condition and in the age of the animals. No consistent trend appeared in relation to the latter conditions except that barnacles in the nauplius stage were killed much more quickly than the adults, as noted below.

For both *B. balanoides* and *B. eburneus*, the killing time ranged between two and five days for concentrations of 0.60 to 1.00 mg. Cu/liter. At 0.22 mg./liter the animals succumbed after ten days. At concentrations of 0.06 mg./liter and less, the animals remained alive for two weeks or more. When the reciprocal of the killing time was plotted against the concentration, as in Figure 1, a roughly linear relation was found. The approximate linearity of this relation indicates that the rate of action of the poison is directly proportional to its concentration.

The results of a series of tests on the poisoning of nauplii, made on the day of their release from the brood pouch, are shown in Table II. They indicate that the

TABLE II

*Effect of concentration of cupric citrate on the survival of nauplii of Balanus eburneus.*  
August 25-27, 1942

Concentration mg. Cu/liter	After 22 hrs.	After 29 hrs.	After 48 hrs.
1.00	all dead	—	—
0.50	all dead	—	—
0.25	all dead	—	—
0.13	75% alive	all dead	—
0.06	75% alive	50% alive	1% alive
0.03	100% alive	95% alive	5% alive
Control	100% alive	95% alive	5% alive
Control	100% alive	95% alive	10% alive

nauplii are killed after much shorter exposure than is the case with the adults. The test was not very satisfactory because the controls lived for only about two days.

### Mussels

The experiments on the mussel, *Mytilus edulis*, indicate that it is much more sensitive to cupric citrate than the barnacle (Tables III and IV). A half-day exposure to 0.55 mg. Cu/liter was sufficient to kill the three mussels in this concentration. One animal in each of the experiments succumbed following a half-day exposure to a concentration of only 0.14 mg. Cu/liter. With adult barnacles, exposures of two days and five days are required for killing at these concentrations. In these tests, and in many others with mussels, death did not ensue until several days after the animals had been transferred to fresh sea water. In one case the mussel did not die until the tenth day following a half-day exposure to the poison. In most cases the animals showed no outward sign of unhealthy condition in the interim.

TABLE III

*Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test three animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 6, 1942*

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.55	$\frac{1}{2}$	4, 8, 10
	1	2, 3, 4
	2	2, 3, 3
	3	2, 3, 3
0.14	$\frac{1}{2}$	4, *, *
	1	3, 3, 4
	2	3, 4, 4
	3	3, 4, 4

\* Still alive when experiment terminated on August 18.

TABLE IV

*Relation of killing time to concentration of cupric citrate in the mussel, *Mytilus edulis*. In each test two animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 10, 1942*

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.14	$\frac{1}{2}$	5, *
	1	3, $\frac{1}{2}$
0.08	$\frac{1}{2}$	*, *
	1	*, *
	2	6, 8
0.04	$\frac{1}{2}$	*, *
	1	*, *
	2	*, *
	3	8, *
0.02	1	*, *
	2	*, *
	$\frac{1}{2}$	*, *

\* Still alive when experiment terminated on August 20.

#### *Accumulation of copper in barnacle tissues during poisoning and recovery*

Several series of experiments were undertaken to determine the extent to which copper accumulated in the tissues of the barnacles subjected to solutions of cupric citrate. Since the shell material of the barnacle is several times greater in volume and many times greater in dry weight than the soft tissues, a technique was developed for the copper assay of both constituent parts of the animal separately. The number of barnacles used for a single analysis varied from 15 to 40 with the size and expected copper content. In the earlier experiments, the animals were chiseled free from the substratum, but in so doing all the fluid material was lost, and bits of broken shell were sometimes included with the soft parts. In the later experiments the soft tissues were taken from the shell without disengaging the animals from the substratum by removing the operculum and picking out the soft parts with forceps. The fluid material, which was removed with a pipette, and the tissues were then placed in a weighed Erlenmeyer flask. In the case of mussels, a much smaller number of animals was used. The relatively large amount of sea water within the mantle cavity of the mussel was drained off and discarded before removing the soft parts.

The wet weight of the soft tissues of barnacles and mussels was obtained and the approximate volume of the wet tissue was determined by forcing it into a graduated cylinder.<sup>3</sup> The material was transferred quantitatively to a flask, dried in an oven at 100° C., and the dry weight determined. Enough H<sub>2</sub>SO<sub>4</sub> was added to char the material. It was heated to fumes on a hot plate, and concentrated HNO<sub>3</sub> was added dropwise until a colorless solution was obtained. The solution in the flask was transferred quantitatively to a 100 cc. volumetric flask and diluted to the mark. Test for copper was made by the carbamate method (Coulson, 1937). A blank to which the same amount of reagent was added was also carried through the above procedures. In the analysis of barnacle shells, the wet weight of the shells was first

<sup>3</sup> In the early experiments, volume in cubic centimeters alone was determined, but was subsequently found to be very nearly equal to the wet weight in grams.

determined. The shells were then dried in the oven at 100° C. overnight and the dry weight determined. The material was ignited in the muffle furnace at 800°–900° C. for three hours, removed and cooled. Two cubic centimeters of concentrated HCl were added, warming if necessary to dissolve.\* The remainder of the analysis followed the same procedure as with the soft tissues.

Determinations revealed that the amount of copper in the shell tissue of both normal and poisoned barnacles was quite variable. There was also no consistent change in the copper content of the shells in the case of batches of poisoned barnacles with increasing amounts of copper in the soft tissues. Accordingly, in the later experiments the laborious and rather unsatisfactory copper assay of the shells was omitted.

The copper content of barnacles freshly brought into the laboratory is many times greater than an equal volume or an equal weight of sea water (Table V). Evidently under natural conditions, the barnacle tends to accumulate copper in its tissues to a considerable extent.

TABLE V  
Copper content of freshly collected barnacles  
Woods Hole sea water contains 0.00001 to 0.00003 mg./cc.

Species	Source	Number	Copper in soft parts			Copper in shells		
			mg.	mg./cc.*		mg.	mg./cc.	
<i>Balanus eburneus</i>	Cotuit, July 28	50	0.0017	0.0014		0.141	0.027	
	Cotuit, July 28	50	0.0027	0.0024		0.097	0.014	
			mg.	mg./g. Wet wt.	mg./g. Dry wt.	mg.	mg./g. Wet wt.	mg./g. Dry wt.
<i>Balanus eburneus</i>	Cotuit, Aug. 20	50	0.015	0.0038	0.008	0.007	0.0003	0.0004
	Cotuit, Aug. 20	40	0.010	0.0019	0.014	—	—	—
	Cotuit, Sept. 12	30	0.016	0.0011	0.016	—	—	—
<i>Balanus balanoides</i>	Woods Hole, July Buzzards Bay	100	0.029	0.029	0.104	0.069	0.0070	0.0075
		50	0.023	0.027	0.068	0.063	0.0065	0.0067

\* May be assumed equivalent to mg./g. wet weight.

In experiments designed to test whether barnacles could accumulate copper in their tissues, it was found that the amount of copper present increased rapidly for each day that the animals remained in the toxic solution. At the end of four days of continuous poisoning in the two concentrations used, all the animals were dead (Blocks A and C, Tables VI and VII). However, in those cases in which the barnacles were transferred to fresh sea water after one or two days, the animals were restored to what appeared to be a healthy condition.<sup>5</sup> Analysis of the tissues of the barnacles which had been replaced in fresh sea water showed that the animals had not only recovered their activity but also had eliminated a certain amount of copper. These barnacles still contained, however, as much as twenty-five times the amount of copper found in their tissues before poisoning began.

<sup>4</sup> If an excess of CaCO<sub>3</sub> remained, more acid was added until evolution of CO<sub>2</sub> ceased.

<sup>5</sup> The recovery of Bugula larvae from copper poisoning was reported by Miller and Cupp (1942) and Miller (1946).

TABLE VI

*Changes in copper content of soft tissues during poisoning and recovery**Toxic: Cupric citrate, conc. 0.20 mg. Cu/liter**Test Animal: Balanus eburneus**Date: August 3-8, 1942**Basis: Copper content per cubic centimeter of soft tissue \**

Block A		Block B	
Initial copper content	0.010 mg.	Initial copper content	0.010 mg.
After 1 day in toxic	0.034 mg.	After 1 day in toxic	0.034 mg.
After 2 days in toxic	0.070 mg.	After 1 day in sea water	0.033 mg.
After 3 days in toxic	0.093 mg.	After 2 days in sea water	0.030 mg.
After 4 days in toxic	0.112 mg.	After 3 days in sea water	0.035 mg.
		After 4 days in sea water	0.026 mg.
Condition at end—dead		Condition at end—open, inactive but reacts to touch	

\* May be assumed equivalent to wet weight.

TABLE VII

*Changes in copper content of soft tissues after varying exposures to toxic and after recovery**Toxic: Cupric citrate, conc. 0.35 mg. Cu/liter**Test Animal: Balanus eburneus**Date: August 10-17, 1942**Basis: Copper content per cubic centimeter of soft tissue \**

Block C		Block D		Block E	
Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.
After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.
After 2 days in toxic	0.073 mg.	After 2 days in toxic	0.073 mg.	After 1 day in sea water	0.029 mg.
After 3 days in toxic	0.089 mg.	After 1 day in sea water	0.056 mg.		
After 4 days in toxic	0.075 mg.	After 9 days in sea water	0.054 mg.	After 6 days in sea water	0.022 mg.
Condition at end—dead.		Condition at end—cirri give incom- plete sweep or full sweep but slow.		Condition at end—normal rapid sweep of cirri.	

\* May be assumed equivalent to wet weight.

These indications that barnacles can take up relatively large quantities of copper without being killed and can eliminate the poison to a certain extent were confirmed by more elaborate experiments which were designed primarily to test the effects of the toxic and of different periods of exposure and of recovery (Tables VIII to XI).<sup>6</sup> These experiments demonstrated that barnacles took up copper

<sup>6</sup> In these experiments the dry weight of the soft tissues to be analyzed was determined and the copper content stated on the more accurate basis of milligrams of copper per gram of dry weight. The dry weight for the soft parts of the barnacle was found to be about 5 per cent to 20 per cent of the wet weight. Since this group of experiments was conducted during late summer and autumn, there exists the possibility that the results may not be strictly comparable with the earlier work. The barnacles tested were necessarily older and larger, and were no doubt in a different physiological condition. It was noted that the animals became less active as the season progressed, and by late autumn they tended to remain closed for long periods. This last fact alone would be expected to cause an important difference in the rate at which toxic was absorbed. In general, the animals tended to survive longer in high concentrations of toxic in the autumn than they had in the summer.



TABLE VIII

*Comparison of changes in copper content of soft tissues during continuous exposure to low concentration of toxic and intermittent exposure to higher concentration*

*Toxic: Cupric citrate, Block F: 0.14 mg. Cu/l.; Block G: 0.22 mg. Cu/l.*

*Test Animal: Balanus eburneus*

*Date: August 24 to September 28, 1942*

*Basis: Copper content per gram of dry weight of soft tissue*

*Exposure times indicated for toxic are cumulative totals*

Block F		Block G	
Initial copper content	0.014 mg.	Initial copper content	0.008 mg.
After 1 day in toxic	0.05 mg.	After 1 day in toxic	0.037 mg.
After 2 days in toxic	0.08 mg.	(Replaced in sea water for 1 day)	—
After 7 days in toxic	0.33 mg.	After 2 days in toxic	0.095 mg.
After 15 days in toxic	1.09 mg.	(Replaced in sea water for 1 day)	—
(Replaced in fresh sea water)		After 3 days in toxic	0.18 mg.
After 18 days in sea water	0.39 mg.	(Replaced in sea water for 6 days)	—
		After 4 days in toxic	0.23 mg.
			(0.19 mg.)*
		(Replaced in sea water for 7 days)	0.17 mg.
		After 7 days in toxic	0.48 mg.
		(Replaced in sea water for 16 days)	0.51 mg.
Condition at end—full but slow sweep of cirri		Condition at end—full but slow sweep of cirri	

\* Batch of dead barnacles.

faster from the more concentrated toxic media. With the slower rate of absorption at the lower concentrations, the animals appeared to withstand higher amounts of copper in their tissues. Thus the highest copper content observed for living barnacles, namely, 1.09 mg. per g. dry weight, was found after fifteen days in 0.14 mg. Cu/liter cupric citrate (Table VIII, Block F). In cases in which large amounts of copper were absorbed the barnacles tended to live longer and to remain in better condition when periods in sea water were alternated with periods in the toxic medium.

In cases where the copper content was determined for the dead barnacles, which were found among the living individuals in increasing number as the experiment progressed, it was almost invariably true that the amount of copper present was less than that for the living specimens from the same block. This observation suggests that the entrance of copper and its fixation in the tissues of the barnacle is not simply a mechanical process of diffusion or adsorption but is a process which is influenced in some way by the metabolic reactions of the living organisms.

Due to the facts considered above, it is not possible to arrive at any definite value for an absolute amount of copper which must be absorbed by the barnacle to kill it, nor at any rate of absorption above which death will ensue. The results suggest that killing may not be due to the destruction of some vital substance or process by the direct action of the copper as much as to a general depressing action of the toxic on some function, such as the feeding reaction, to the extent that death eventually follows.

TABLE IX

*Comparison of changes in copper content of soft tissues during continuous and intermittent exposures to high concentration of toxic and continuous exposure to lower concentration*

*Toxic: Block H—Cupric citrate 0.75 mg. Cu/l. Continuous*

*Block I—Cupric citrate 0.35 mg. Cu/l. Continuous*

*Block J—Cupric citrate 0.75 mg. Cu/l. Alternating daily with sea water*

*Test Animal: Balanus eburneus*

*Date: September 21 to October 14, 1942*

*Basis: Copper content per gram of dry weight of soft tissue*

Block H		Block I	
Initial Cu content	0.016 mg.	Initial Cu content	0.016 mg.
After 2 days in toxic	0.17 mg.	After 3 days in toxic	0.13 mg.
After 4 days in toxic	0.30 mg.	After 6 days in toxic	0.25 mg.
After 6 days in toxic	0.38 mg.	After 9 days in toxic	0.32 mg.
After 7 days in toxic	(0.33 mg.)*	After 11 days in toxic	0.76 mg.
After 8 days in toxic	0.48 mg.	After 14 days in toxic	(0.39 mg.)*
After 11 days in toxic	(0.84 mg.)*	After 18 days in toxic	0.49 mg.
		After 18 days in toxic	(0.43 mg.)*
		After 18 days in toxic	0.60 mg.
Block J			
Initial Cu content		0.016 mg.	
After 2 days in toxic, 1 day in sea water		0.16 mg.	
After 4 days in toxic, 3 days in sea water		0.13 mg.	
After 6 days in toxic, 6 days in sea water		0.31 mg.	
After 8 days in toxic, 7 days in sea water		0.42 mg.	
After 8 days in toxic, 8 days in sea water		0.25 mg.	
After 10 days in toxic, 9 days in sea water		0.48 mg.	
After 12 days in toxic, 11 days in sea water		0.50 mg.	
After 12 days in toxic, 11 days in sea water		(0.48 mg.)*	

\* Batch of dead barnacles.

TABLE X

*Changes in copper content of soft tissues during recovery from various exposures to toxic*

*Toxic: Cupric citrate 0.75 mg. Cu/l.*

*Test Animal: B. eburneus*

*Date: October 21–November 12, 1942*

*Basis: Copper content per gram of dry weight of soft tissue*

	Treatment	Copper content	Condition
Block L	After 3 days in toxic	0.38 mg.	++
	After 10 days in sea water	0.31 mg.	+
	After 14 days in sea water	0.21 mg.	+
Block M	After 6 days in toxic	0.38 mg.	+
	After 7 days in sea water	0.36 mg.	+
	After 14 days in sea water	0.40 mg.	++
Block N	After 9 days in toxic	0.58 mg.	0+
	After 9 days in toxic	(0.39 mg.)	dead individuals
	After 7 days in sea water	0.43 mg.	+
	After 14 days in sea water	0.39 mg.	++

No barnacles were found to have been killed by the absorption of less than 0.19 mg. of copper per g. of dry weight—an amount which is ten or more times the normal content of the tissues. In many cases animals succumbed after about 0.4 mg. per gram of dry weight had been taken up by the tissues. In no case did the barnacles absorb more than 1.09 mg. of copper per gram of dry weight. Barnacles which had accumulated copper from toxic solutions to the extent of 0.5 mg. to 1.09 mg./g. dry weight, in some cases revived when replaced in fresh sea water and were still alive two or three weeks later. At the end of the period, when they had regained normal, or nearly normal, activity, they contained 0.3 to 0.5 mg. of copper per g. of dry

TABLE XI

*Changes in copper content of soft tissues during recovery from exposures at high concentrations of toxic*

*Toxic: Block O—Cupric citrate 0.90 mg. Cu/l.*

*Block Q—Cupric citrate 0.75 mg. Cu/l.*

*Test Animal: B. eburneus*

*Date: October 21–November 21, 1942*

*Basis: Copper content per gram of dry weight of soft tissue*

	Treatment	Copper content	Condition
Block O	After 8 days in toxic	0.52 mg.	0+
	After 8 days in toxic	(0.48 mg.)	dead individuals
	After 8 days in sea water	0.42 mg.	0+
Block Q	After 9 days in toxic	0.53 mg.	0+
	After 9 days in toxic	(0.35 mg.)	dead individuals
	After 8 days in sea water	0.40 mg.	+
	After 15 days in sea water	0.35 mg.	++

weight. In most instances the barnacles eliminated copper from their tissues, especially in cases where a high concentration had been accumulated. Although the copper which had been absorbed was never entirely eliminated, and although the process was slower than for accumulation, the experiments show that barnacles have some ability to rid their tissues of copper.

#### *Accumulation of copper in mussel tissue during poisoning and recovery*

To determine the amount of copper taken up by the tissues of the mussel when subjected to copper solutions, groups of 25 mussels were placed in various concentrations of cupric citrate and were later transferred to fresh sea water after increasing periods of time. The media were renewed every day. The copper content of 20 fresh animals from the same source was assayed at the beginning of the experiment and batches of three to five animals were withdrawn from the various groups for copper assay after the indicated number of days during poisoning and during recovery (Table XII).

The initial copper content of the mussel tissue was 0.0109 mg. per gram of dry weight, roughly the same as for the barnacle. Since the wet weight of the soft parts of the mussel was similarly found to be about ten times the dry weight, it is

clear that the fresh mussel also contains many times the concentration of copper present in the sea water.

After the first day in the toxic media, the mussels in the more concentrated solutions had taken up six to eight times their original amount of copper, but in the weaker solutions, there was no consistent increase in copper content until after an exposure of three days. The group of mussels (Group 4) in the weakest solution had absorbed only about two and one-half times their initial copper content after five days—or less than half the amount the Group 1 mussels had absorbed in one day in a solution five times as strong. In addition, the mussels were found to be able to rid their tissues of copper effectively. One day's sojourn in fresh sea water was suffi-

TABLE XII

*Changes in copper content of soft tissues of mussel during poisoning and recovery*

*Toxic: Cupric citrate*

*Test Animal: Mytilus edulis, 25 animals (5 cm. long) in each group*

*Date: August 19 to September 5, 1942*

*Basis: Copper content in milligrams per gram of dry weight of soft tissue*

*Exposure to toxic solution began August 19. The line marks the time when mussels were transferred to fresh sea water*

Group	Concentration of Cu citrate mg. Cu/l.	No. of days in toxic	Content on indicated date							
			Aug. 20	Aug. 21	Aug. 22	Aug. 23	Aug. 24	Aug. 25	Aug. 26	Sept. 5
1	0.120	1	0.066	0.0196	—	—	—	—*	—	—
2a	0.082	1	—	0.0135	—	—	0.017	—	—	0.0085
2b	0.082	2	0.084	0.031	0.023	—	—	—	—	0.0077
3	0.049	3	0.0076	—	0.024	0.018	—	—	0.017	0.0100
4	0.027	5	0.0135	—	0.021	—	0.025	0.029	—	0.0130
Control	0	0	0.0109	—	—	—	—	—	—	—

\* Condition on August 25: Group 1—all dead  
Group 2a—9 dead  
Group 2b—10 dead  
Group 3—3 dead  
Group 4—4 dead

No further deaths to September 5.

cient to reduce the amount of copper in the mussels from the strongest solutions by more than 70 per cent. In cases where longer exposures were employed (although in lower concentrations) more time seemed to be required for the elimination of the copper—perhaps because it had penetrated to deeper tissues. At the end of about two weeks in sea water, the copper content of the mussel tissue was back to normal—a fact quite in contrast to the situation with the barnacle.

In spite of the fact that after one day in sea water the copper content of the Group 1 mussels had been reduced at least to the amount reached by the mussels in the weaker solutions, all the animals in this group died within five days. Some irreparable damage had been done by the single day's exposure to the high concentration of the toxic. In the lower concentrations only three to four animals died

despite exposures to the toxic for three to five times as long. It is possible that the mussels in the lower concentrations could eliminate copper from vital tissues as fast as it tended to enter.

Although the values obtained in this single experiment are somewhat irregular, two important differences are indicated by the results as compared with the results obtained with barnacles. At the same concentrations, the exposures which are lethal to mussels are considerably shorter than for barnacles. Similarly the mussels succumbed after accumulating amounts of copper in their tissues which were much lower than was the case with barnacles.

#### TESTS ON THE POISONING OF CYPRIDS

During January, 1943, experiments were undertaken at Miami Beach, Florida, on the poisoning of barnacles in the cyprid stage. Especial interest centered on these observations because the cyprid is the stage in which the animal first attaches itself to the substratum. It is the opinion of many that once a barnacle becomes attached, it can be killed only with great difficulty and that even if it is killed, the shell does not drop off but remains firmly attached. Actually, attachment takes place in two steps: first, the attachment of the antennae of the cyprid, following which metamorphosis is begun; and second, the attachment of the base of the barnacle after metamorphosis has been completed.

Cyprids were obtained by suspending glass microscope slides in Biscayne Bay overnight. The cyprids were presumed to be those of *Balanus improvisus*, which is the commonest adult barnacle in the neighborhood. The slides were brought into the laboratory where the following details in the development of the cyprids were observed.

The cyprids are originally attached by their antennae. About 5 or 6 hours after attachment, the animal undergoes metamorphosis in the course of which the cyprid shell is moulted and the animal emerges as a more or less round body still adhering to the substratum by the original attachment which now appears as the center of the convex base of the animal.

As development proceeds the base of the animal, which was at first freely movable, tends to flatten and to be pressed against the substratum. About 24 hours after metamorphosis the base is completely flattened with relatively sharp edges. A pulsation of the central parts of the animal has begun by this time, and the operculum may open, but no cirri are extended. The base is not caused to move by the activity of the animal, as before, but any part of it, except the original central point of attachment, is easily dislodged by pressure with a needle.

After another 24 hours, the cirri may be extended and filter actively, and the main area of the base begins to adhere to the substratum. At first the base merely gives the impression of being sticky; later it becomes attached with sufficient firmness so that it cannot be dislodged without tearing the tissue. Not until the third day or later does hard material (presumably calcium carbonate) begin to appear in the base.

The attachment of the cyprid by its antennae takes place quickly, and the original attaching structure remains in existence at least until calcification begins. The attachment of the base of the newly metamorphosed barnacle is a slow process which provides only weak adhesion at first but which finally supplies a permanent and

TABLE XIII

*Effect of different concentrations of cupric citrate in preventing the metamorphosis of barnacle cyprids attached to glass slides*

*Species: probably Balanus improvisus*

Concentration mg. Cu/liter	Number of cyprids exposed	Number of cyprids which metamorphosed
116.	6	2 (moult incomplete)
58.	6	3 (moult incomplete)
23.	4	4
9.7	3	3
4.9	2	2
0.93	11	11
0.47	9	9
0.23	5	5
0.12	9	9

firmly cemented calcareous structure. Poisoning of the barnacle could theoretically be accomplished either in the attached cyprid stage or as a newly metamorphosed animal before the calcification of the base.

The susceptibility of the cyprid to poisoning was investigated by placing cyprids attached to glass slides in solutions of cupric citrate (Table XIII) and of mercuric chloride (Table XIV). In the case of the copper solutions, the cyprids metamorphosed successfully in concentrations as great as 23 mg. Cu/l and solutions as strong as 116 mg. Cu/l were only partially inhibitory. Concentrations about 100 times greater than those ordinarily tolerated by adult barnacles, therefore, were not capable of killing the cyprids before they completed their metamorphosis.

In the case of the mercury solutions, although an inhibitory effect was observed at a lower concentration (16.6 mg. Hg/liter) than for copper, much higher concentrations of the toxic were required to prevent metamorphosis than would ordinarily kill adult barnacles.

Failure to kill the cyprid may be due to an unusually high resistance of the animal to poison in this stage or to the relative shortness of the period between attachment and metamorphosis, or both. From these results it is probable that the metamorphosis of the attached cyprid cannot be stopped by any concentration of toxic which could practically be obtained from a paint.

TABLE XIV

*Effect of different concentration of mercuric chloride in preventing the metamorphosis of barnacle cyprids attached to glass slides*

*Species: probably Balanus improvisus*

Toxic solution mg. Hg/liter	Number of cyprids exposed	Number which metamorphosed
83.	5	0
16.6	6	3 (moult incomplete)
3.3	6	6
0.66	9	9
0.17	7	7
0.09	10	10

The susceptibility to poisoning of the newly metamorphosed barnacle was tested in a similar fashion by placing slides bearing animals in this stage in solutions of cupric citrate (Table XV). Much lower concentrations of copper were required to kill the newly metamorphosed barnacle than was necessary to prevent the cyprids from carrying out their metamorphosis. The relation between killing time and concentration is roughly of the same order of magnitude as observed for adult barnacles at Woods Hole. There is some indication, however, that the newly metamorphosed animals are slightly more resistant, as concentrations of 0.23 mg. Cu/l to 0.47 mg. Cu/l were required to kill in five days in contrast to concentrations of only 0.14 to 0.30 mg. Cu/l for the older animals.

Although recently metamorphosed barnacles survived for several days in concentrations of cupric citrate of 0.12 mg. Cu/l or lower, their development was very materially retarded. In the stronger solutions the base remained in the rounded

TABLE XV

*Relation of killing time to concentration of cupric citrate in barnacles  
which had just completed metamorphosis*

*Species: probably Balanus improvisus*

Concentration mg. Cu/liter	Killing time days
9.7	1
4.9	1-2
0.97	3
0.93	3-4
0.47	5
0.23	5
0.20	7
0.12	10

condition, and in concentrations down to at least 0.20 mg. Cu/l the base never became calcified nor rigidly cemented to the substratum, although it might become flattened and adhesive.

In a small number of tests reported subsequent to this investigation by Pyefinch and Mott (1944), free swimming cyprids of *Balanus balanoides* were killed in 24 hours by 0.5 to 1.0 p.p.m. copper from cupric sulphate. Metamorphosis was completed in concentrations up to 7 p.p.m. but after metamorphosis the barnacles were killed in 3 days by 0.5 p.p.m. Mercury from mercuric chloride was found to be more toxic than copper. These results agree very satisfactorily with ours.

At still lower concentrations of copper, in the present experiments, a complete change in the effect of the metal evidently comes about, for development was accelerated. Animals in the lowest concentration tested, namely 0.06 mg. Cu/l, reached the stage of active filtering with their cirri in less than 48 hours after metamorphosis, although the controls did not attain this stage until about 2 days later. It appears that a small amount of copper acted as a stimulant to development, and that larger amounts of copper retarded development even though they may be insufficient to kill the animals outright.

## DISCUSSION

Adult barnacles, and perhaps young stages also, can absorb ten to possibly one hundred times as much copper as their tissues normally contain without apparent injury. They can eliminate a certain amount of this material also. Therefore, when copper is used as a toxic in an antifouling paint, a substance is employed which can be taken up with impunity in relatively large quantities by the barnacle and to a lesser extent by the mussel. The animals can recover from the effects of this poison, to a certain extent at least, when again bathed by fresh sea water.

In some cases mussels which had received a lethal dose of poison remained in an apparent healthy condition for several days after being replaced in fresh sea water before dying. No such latent period was observed in the experiments with barnacles.

Possibly related to the facts just considered is the indication that the action of copper as a poison is not so much the direct destruction of some tissue or vital material in the barnacle as a general retardation of life processes. In the case of the adult barnacle, subjection to copper solutions causes a slowing and eventual cessation of the filtering activity of the cirri. In the case of the newly metamorphosed stages, not only is movement inhibited but development is seriously retarded as well. Much more satisfactory as a poison would be some substance which struck directly and irreversibly at some vital point in the animal and which could not be eliminated from the tissues. With such an ideal poison, barnacles which received only small doses, due to unavoidable dilution, would eventually be killed when a lethal amount of the material had accumulated. We have shown that with copper, sublethal doses may possibly stimulate development and certainly may allow subsequent recovery. Evidence that copper may stimulate the attachment of larvae has been reported by Prytherch (1934) for the oyster and by Miller (1946) for *Bugula*.

The relative vulnerability of the various steps in the attachment and growth of barnacles has been revealed by this investigation. The original idea that barnacles could be eliminated from a ship's hull only by preventing their initial attachment is partly right and partly wrong. As explained above, the attachment actually takes place in two steps. There is little promise of using poison successfully to prevent the first step, namely the attachment of the cyprid by its antennae, since this apparently takes place in a matter of minutes. However, we should not lose sight of the very important, but as yet unrealized, possibility of finding a substratum toward which the cyprid would display an avoiding reaction or to which the cement of the antennae would not adhere.<sup>7</sup>

Our tests have demonstrated the practical impossibility of preventing by poison the metamorphosis of the cyprid or of killing it during the process. This conclusion is substantiated by the results of Pyefinch and Mott (1944) and is in agreement with their statement: "Settlement and metamorphosis can take place on a paint in other respects anti-fouling though death of the barnacle occurs after metamorphosis." Similarly, at the other end of the life cycle, after the adult barnacle has attained any considerable size, it can be poisoned only with great difficulty for the reasons already discussed.

<sup>7</sup> In certain tests under laboratory conditions, Pyefinch and Mott (1944) found that cyprids failed to settle in concentrations of copper as low as 0.03 p.p.m.



There remains, however, the possibility of effectively preventing the second step in the attachment process, namely the permanent cementing of the base of the newly metamorphosed animal to the substratum. Although a long exposure to copper or a high concentration is necessary to kill the barnacle outright at this stage, moderate concentrations of poison tend to retard development and possibly to prevent the formation of the calcareous base, or the cement for this base, with the result that the young barnacle never succeeds in establishing a firm attachment, and hence is eventually displaced. The newly metamorphosed stage, therefore, appears to be the most vulnerable to copper poisoning.

The indication is, then, that copper paints act by preventing attachment but do so chiefly during the second step of the process through interference with the final cementing of the base of the metamorphosed barnacle to its substratum.

#### SUMMARY

1. Direct tests were performed on the concentrations and exposures of a variety of metallic salts necessary to kill barnacles. The toxicities of mercury, cupric citrate, cupric tartrate, cupric salicylate, and cupric aminobenzoate were found to be slightly less than the toxicity of basic cupric carbonate. The toxicity of silver is about equal to that of basic cupric carbonate, but the toxicity of zinc is very much less.

2. The rate of killing of barnacles by cupric citrate is proportional to the concentration of the toxic over the range tested.

3. An extremely high concentration of copper or of mercury salts was necessary to prevent the metamorphosis of cyprids attached to glass plates. The results show the difficulty of preventing the *initial* attachment of cyprids, or their metamorphosis, by the use of copper paints.

4. Moderate concentrations of cupric citrate seriously retard the development of the newly metamorphosed barnacles and prevent the second step in attachment, namely, the formation of the cemented calcareous base.

5. Exposure of the newly metamorphosed barnacles to very low concentrations of cupric citrate accelerated development beyond that of the normal animals.

6. The soft tissues of adult barnacles normally contain a much higher concentration of copper than does sea water. When placed in solutions of cupric citrate, barnacles absorbed more than 100 times the normal copper content of the tissues. In no case were barnacles killed by the absorption of less than 0.19 mg. of copper per gram of dry weight—more than 10 times the normal content. In some cases barnacles which had absorbed 0.5 mg. to 1.09 mg./g. from toxic solutions revived when replaced in fresh sea water.

7. It was demonstrated that when replaced in fresh sea water, barnacles can eliminate from their tissues as much as two-thirds of the copper which has been absorbed from toxic solutions.

8. Mussels are more sensitive to poisoning by cupric citrate than barnacles. When exposed to copper solutions, mussels take up copper more rapidly than do barnacles, and when replaced in fresh sea water, they eliminate it from their tissues more rapidly and extensively. In many cases in which a considerable portion of the copper was eliminated, the mussels nevertheless succumbed subsequently.

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INSTITUTE LIBRARY SINCE FEBRUARY, 1943<sup>1</sup>

EXPLANATION OF SYMBOLS

Titles recorded alphabetically with initial dates.

- \*   ceased publication.  
[ ]   incomplete.  
+   receive currently.

Abhandlungen der Medizinischen Fakultät  
der Sun Yatsen Universität 1929: Canton.  
2, no. 2; 3, no. 2

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## EFFECTS OF HYDROGEN PEROXIDE PRODUCED IN THE MEDIUM BY RADIATION ON SPERMATOOZOA OF ARBACIA PUNCTULATA

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In the course of a previous investigation (Evans et al., 1941, 1942a) it was found that roentgen radiation exerted effects on *Arbacia* sperm indirectly by producing a very temporary toxicity of the medium. The intermediary appeared to be the "activated water molecule," as postulated by Fricke (1935), and was characterized by the following phenomena. Sperm irradiated in sea water suffered immediate reduction in fertilizing capacity, whereas sperm irradiated "dry" were apparently unaffected in this respect. The smaller the concentration of sperm in the suspension during irradiation the greater the percentage made incapable of fertilization. Presence of proteins, such as egg albumin, afforded "protection," that is, greatly increased the percentage of sperm surviving irradiation. Unirradiated sperm did not suffer immediate reduction in fertilizing capacity when placed in water that had been exposed to amounts of radiation capable of producing such effects when the sperm were present during the irradiation. Under certain conditions "activated water" reacts to produce hydrogen peroxide (Risse, 1930; Taylor et al., 1933; and Fricke, 1934a) which is toxic to organisms and may account for some or all of the indirect effects of X-rays. To understand better the indirect action of X-rays on cells, comparative studies of the effects of irradiated water and hydrogen peroxide were made on *Arbacia* sperm and ova.

### MATERIALS AND METHODS

Male *Arbacia* were stimulated, by cutting through the oral region, to shed sperm in a dish of sea water. The suspension was centrifuged until the sperm were closely packed, then the supernatant fluid was removed. Before placing the sperm in test solutions, nine parts of sea water were mixed with one part of packed sperm in order to facilitate transfer of equal amounts of sperm suspension to the various dishes. One part of this suspension was added to ninety-nine parts of the test solution making a final concentration of 1:1,000 sperm. Percentage fertilizing capacity was determined by inseminating a given lot of freshly collected eggs with as many drops of control sperm as needed to produce 90-99 per cent of the eggs with

<sup>1</sup> It is a pleasure to express appreciation to Dr. G. Failla for his helpful criticisms and to Miss Anne R. Behan for technical assistance.

fertilization membranes. The same volume of sperm from the experimental medium was then tested with a lot of eggs similar to those used for the controls.

Samples of sperm from sea water and from experimental media were taken at regular intervals and tested for fertilizing capacity. The survival period was taken as the time required to reduce the percentage fertility of a given lot of sperm to less than 50 per cent. The actual survival time was somewhat longer in each case as some sperm would still be alive after the suspension had completely lost the ability to fertilize untreated eggs. A few observations were made of longevity as indicated by rate of oxygen consumption and motility of sperm. The relative decrease in survival time of different experimental lots was approximately the same whether these criteria or fertilizing capacity were employed. Sperm in a concentration of 1:10 suffered no change in fertilizing capacity during the time covered by these experiments. Therefore, when it became necessary to employ a new batch of eggs, the proper number of drops of sperm suspension to use in each test was determined by employing a freshly diluted 1:1,000 suspension. Cleavage time was taken as the number of minutes (at 25° C.) from the time of insemination until 50 per cent of the fertilized eggs had undergone division. Catalase solutions used were sea water dilutions of a stock extracted from sperm by distilled water. Albumin solutions were 0.1 gram per cent of powdered egg albumin made up in sea water and used immediately.

Fresh filtered sea water was irradiated in covered plastic dishes at 22–28° C. The material was cross-fired (from above and below) between two water-cooled X-ray tubes operated at 182 kv pk., and 25 ma (inherent filtration equivalent to approximately 0.2 mm. Cu). The intensity was usually 5,600 r per minute and the irradiation was given in one treatment.

## EXPERIMENTAL RESULTS

### *Effects of irradiated water*

Results of exploratory experiments (Evans et al., 1942a; Evans, 1942) indicated that irradiated water reduced the survival time and prolonged the cleavage time of sperm placed therein. The amount of effect varied greatly from one lot of sperm to another. It appeared in general that irradiation below 50,000 r was ineffectual, around 100,000 r was definitely effective, and a dose of 224,000 r was near the optimum in affecting the water so that it reduced survival time of sperm. The delay in first cleavage (treated sperm + untreated ova) was noticeable when only low dosages (below 100,000 r) had been given to the water, but the effect did not appear to increase rapidly as the irradiation became greater. In the exploratory experiments chemical tests indicated presence of hydrogen peroxide in the heavily irradiated water, and this agent was also found to affect survival time and cleavage time of sperm. It was now of interest to determine quantitatively how much of the toxicity of the irradiated water should be attributed to hydrogen peroxide.

It was found that consistent results were obtained when sperm from several males were pooled so that successive experiments were made from the same original collection. The data are shown in Figure 1 and Figure 2. It may be seen in Figure 1 (top graph) that, in all four experiments, the water became more toxic as the amount of radiation was increased. Reduction in survival time by doses below

85,000 r was not very definite. The effect increased rapidly from 85,000 r to 140,000 r and increased, though less rapidly, from 140,000 r to 230,000 r. The latter finding may indicate that equilibrium was being reached between the rate of hydrogen peroxide formation and the rate of destruction by the radiation.

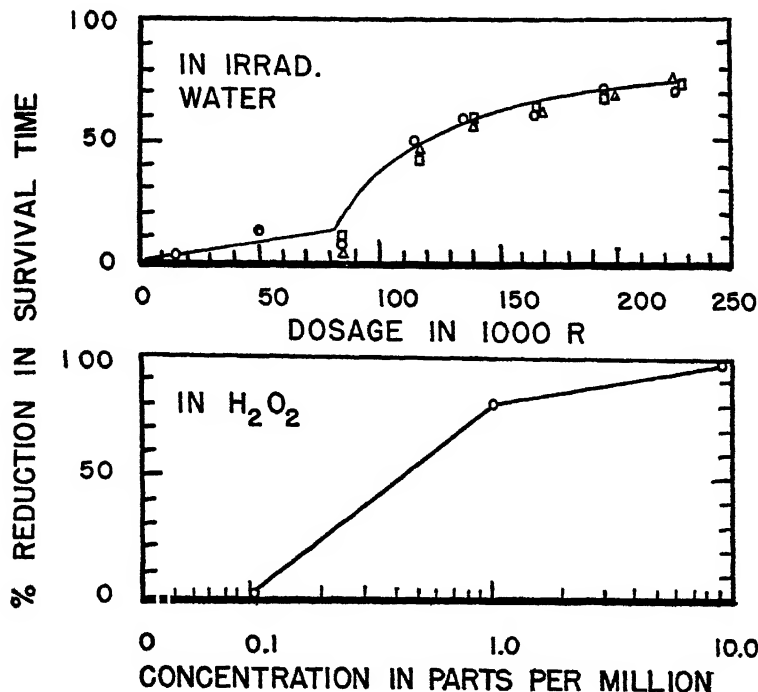


FIGURE 1. Upper graph: effect of different amounts of radiation on the toxicity of sea water to sperm. Toxicity is indicated by a reduction of the survival time (as measured by fertilizing capacity) of the sperm placed in the irradiated water as compared to the survival time of sperm in control sea water.

Lower graph: toxicity of different amounts of hydrogen peroxide in sea water to sperm placed therein.

The effect of retarding cleavage was greater as the irradiation of the water was increased (upper graph of Fig. 2). Also, the cleavage time was more delayed the longer the sperm had been in the irradiated water at the time of insemination. This is shown in the lower graph of Figure 2. The effect approached a maximum, at this concentration of the agent, in about an hour (at 25° C.).

#### *Effects of hydrogen peroxide*

Calculations based on data available in the literature (Fricke, 1934a, 1934b; Taylor, Thomas, and Brown, 1933) and color tests, using titanous chloride (Evans et al., 1942a), of the irradiated sea water indicated that we were dealing with concentrations of hydrogen peroxide in the neighborhood of 1:1,000,000. The effects of different concentrations of hydrogen peroxide<sup>2</sup> on the survival time are shown

<sup>2</sup> Merck's Superoxol reagent hydrogen peroxide (30 per cent).

in the lower graph of Figure 1. One would judge from a consideration of the two graphs of Figure 1 that the maximum amount of hydrogen peroxide produced in the irradiated water was approximately 1:1,000,000.

Hydrogen peroxide in a concentration of 1:1,000,000 retarded cleavage (lower graph of Fig. 2) and the increase in effect with longer exposures was very similar to that of sea water given an irradiation of 224,000 r.

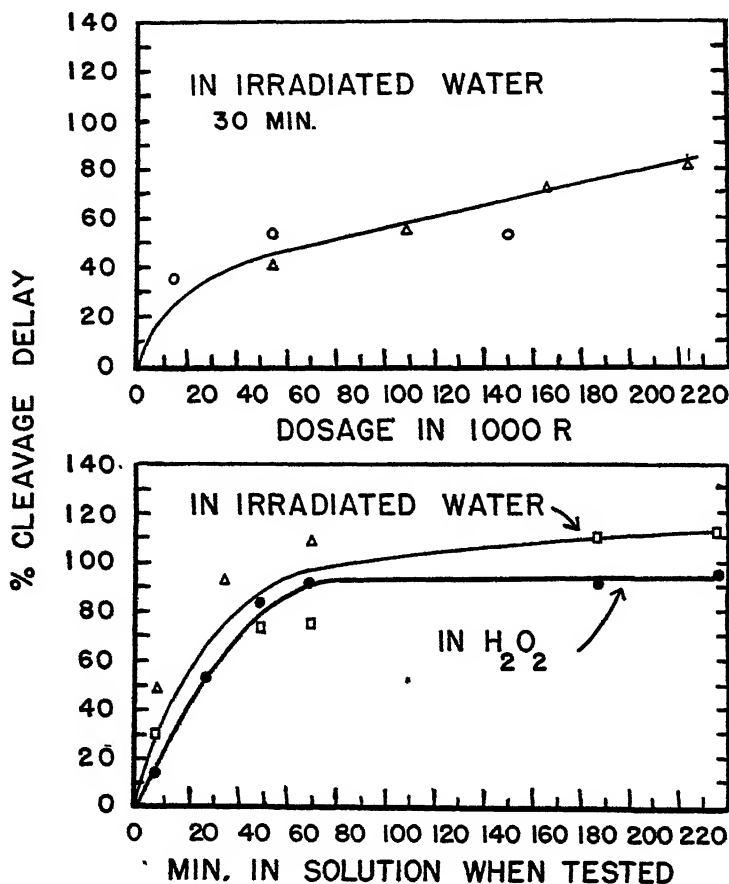


FIGURE 2. Upper graph: effect of different amounts of radiation on toxicity of sea water as indicated by a retardation in time of first cleavage. After sperm had been in the irradiated water for 30 minutes they were used to inseminate fertile eggs in fresh sea water. Cleavage delay is expressed as cleavage time over that of eggs inseminated with control sperm.

Lower graph: effects (on cleavage time) of exposing the sperm for different periods of time to irradiated water (224,000 r) and to water containing hydrogen peroxide (1:1,000,000).

#### *Reduction in toxicity of irradiated water by catalase extract*

It was found that a crude extract possessing catalase activity could be removed from concentrated sperm suspensions.<sup>8</sup> This extract could be added to sperm in

<sup>8</sup> Report in preparation.

sea water without producing any apparent deleterious effects. Results of adding catalase to irradiated water are shown in Tables I and II. It will be seen that the catalase extract completely removed the toxic agent responsible for decrease in survival time (Table I) in all but three instances and greatly reduced the effect in these.

TABLE I  
REDUCTION OF TOXICITY OF IRRADIATED WATER BY CATALASE EXTRACT  
(Percentage decrease in survival time as compared to sperm in control sea water)\*

In irrad. water (160,000 r)	In irrad. water +inactive catalase**	In irrad. water +active catalase	In H <sub>2</sub> O <sub>2</sub> (1:1,000,000)	In H <sub>2</sub> O <sub>2</sub> +active catalase
50	50	8	—	—
92	—	0	90	0
55	55	0	—	—
38	38	0	—	—
81	43	22	—	—
83	48	0	—	—
50	—	0	94	0
81	—	57	—	—

\* There were three control groups (1) sperm in untreated sea water, (2) sperm in sea water plus inactive catalase, and (3) sperm in sea water plus active catalase. There was no decrease in survival time of groups 2 and 3 as compared to group 1. Control time was taken as that of group 1.

\*\* Inactivated by heating (60–65° C.) for 30 minutes.

TABLE II  
REDUCTION IN TOXICITY OF IRRADIATED WATER BY CATALASE EXTRACT  
(Cleavage delay, as compared to controls, in percentage)

Time in experi- mental medium	In irrad.* water	In irrad. water +inactive catalase	In irrad. water +active catalase	In H <sub>2</sub> O <sub>2</sub> (1:1,000,000)	In H <sub>2</sub> O <sub>2</sub> +active catalase
10	36	—	22	22	0
40	37	—	18	31	0
50	45	—	11	27	0
40	48	46	0	—	—
40	45	48	45**	—	—
20	58	55	0	—	—
90	82	88	11	—	—

\* Approximately 160,000 r.

\*\* Sperm introduced immediately after catalase had been added to the irradiated water.

The delay in time of cleavage also was reduced by adding the catalase extract to the irradiated water or to 1:1,000,000 hydrogen peroxide (Table II). Apparently the reason the catalase was not 100 per cent effective in all tests was that in such instances sufficient time was not allowed before the sperm were added to the irradiated water. Maximum efficiency was obtained when the catalase had been in the irradiated water (or hydrogen peroxide solution) for approximately an hour before the sperm were added. In the one test with negative results sperm were added to the irradiated water immediately after the catalase had been introduced, and the

sperm were apparently affected before the  $H_2O_2$  had been destroyed. The amount of catalase used was slightly in excess of that required to remove  $H_2O_2$  (1:1,000,000) as determined in Warburg manometers. It is possible that in some instances the amount of  $H_2O_2$  produced by the irradiation was in excess of 1:1,000,000. Strong concentrations of catalase were avoided as it was found that too much of the extract was toxic to the sperm.

TABLE III  
TOXICITY OF IRRADIATED CATALASE EXTRACT  
(Compared with toxicity of irradiated water)  
(Toxic effects: reduction in survival time and production of cleavage delay)

Irradiation in 1,000 r	% Reduction in survival time		% Cleavage delay	
	Irradiated catalase	Irradiated water	Irradiated catalase	Irradiated water
84	24	10, 5	14	—
112	58	43, 43	20	55
140	74	58, 58	22	—
168	78	62, 62	25	70
196	80	68, 70	30	—
224	83	75, 75	35	80
140	79	—	40	—
140	74	—	30	—
140	74	—	30	—
140	87	55	43	43
140	55 (diluted to 50% with irradiated water)			
100	65	30	40	55
100	65 (diluted to 99% with active catalase)			
140	44 (diluted to 30% with untreated water)			
140	30 (diluted to 30% with active catalase)			

In an earlier investigation (Evans et al., 1942a) it was found that some egg albumin solutions afforded protection against toxicity of irradiated water whereas others did not. In checking over the data it appeared that protection was afforded only when the albumin had been prepared several days previously. Such solutions, on standing, were found to have developed large numbers of bacteria and possessed catalase activity. Fresh albumin solutions had no catalase activity and did not protect the sperm from the toxic action of irradiated water. Fresh albumin did have a protective effect when present with sperm during irradiation (Evans et al., 1942a). The albumin particles apparently competed with the sperm for the limited number of activated water molecules available and thus reduced the amount of injury to the sperm.

#### *Toxicity of irradiated catalase extract*

One type of control in the above experiments (Tables I and II) was the addition of inactive catalase to the irradiated water in which case the toxicity was not reduced. This extract had been made inactive by heating at 60–65° C. for 30 minutes.

It had been found<sup>8</sup> that a dose of 140,000 r would completely inactivate this concentration of catalase, so such an irradiated extract was added to heavily irradiated water to determine whether it would reduce the toxicity. The irradiated catalase failed to reduce the toxicity of the irradiated water, and had even a slightly greater effect in reducing the survival time of the sperm. These results are shown in Table III. Addition of active catalase (two experiments) did not remove the toxicity of the

TABLE IV  
EFFECTS OF IRRADIATED ALBUMIN SOLUTION

Exp. No.	Material	‡ Survival time in min.	Min. cleavage delay
129 Irradiation = 160,000 r	(A) Sperm in control sea water.	70	0
	(B) Sperm in irradiated sea water.	20	15
	(C) Sperm in irradiated sea water + albumin later.	20	20
	(D) Sperm in <i>irradiated albumin solution</i> .	48	22
	(E) Sperm in control sea water + albumin.	1,005	0
20 Irradiation = 224,000 r		(% Survival at 30 minutes)	
	(E) Control water + albumin.	96	0
	(C) Irradiated water + albumin.	59	40
19 Irradiation = 224,000 r	(D) <i>Irradiated albumin</i> .	57	32
	(E) Control water + albumin.		0
	(C) Irradiated water + albumin.		42
21 Irradiation = 64,000 r	(D) <i>Irradiated albumin</i> .		42
	(A) Control water.		0
	(B) Irradiated water.		27
	(F) Irradiated water which had contained 1:100 sperm during irradiation.		5
	(G) Irradiated sperm removed from (F). (Direct effect of irradiation.)		85

radiation-destroyed enzyme extract. Although these results were not conclusive it was indicated that all of the toxicity of the irradiated catalase extract was probably not due to hydrogen peroxide, in other words that some other toxic materials were probably formed. A 0.1 per cent solution of egg albumin was irradiated to determine whether it would be made toxic and the results are shown in Table IV. The tentative conclusion was drawn that the albumin present in the water during irradiation had little effect on the production of hydrogen peroxide by the irradiation, but that albumin itself did not contribute to the toxicity of the solution.

#### *Effects of irradiated water and H<sub>2</sub>O<sub>2</sub> on ova*

Arbacia eggs placed in irradiated water (168,000 r) or in dilute solutions of hydrogen peroxide in sea water exhibited effects similar to those of sperm so treated. These results are shown in Table V. It was found that immersion in irradiated water or in hydrogen peroxide increased the time for first cleavage and decreased the percentage fertilized. It is of interest that the effects were not as pronounced if



the number of eggs in the experimental medium was doubled. This probably indicates that the number of toxic molecules was limited and the eggs were sensitive to even small changes in the concentration of the toxic materials.

TABLE V  
EFFECTS OF IRRADIATED WATER AND HYDROGEN PEROXIDE ON OVA  
(Eggs in experimental medium, inseminated with control sperm)

<i>Experiment 22</i>	Control eggs in sea water	Eggs in irradiated water	Eggs in H <sub>2</sub> O <sub>2</sub> (1:1,000,000)	Eggs in H <sub>2</sub> O <sub>2</sub> (1:100,000)
Cleavage time: (tested after 30 min. in solution.)	42 min.	48 min.	47 min.	
% Fertilization:	100 fert.	73 fert.	100 fert.	55 fert.
Cleavage time: (tested after 90 min. in solution.)	42 min.	49 min.	48 min.	
% Fertilization:	99 fert.	69 fert.	100 fert.	

<i>Experiment 23</i>	N no. eggs in control sea water	2N no. eggs in control sea water	N no. eggs in (1:100,000) H <sub>2</sub> O <sub>2</sub>	2N no. eggs in (1:100,000) H <sub>2</sub> O <sub>2</sub>	N no. eggs in irradiated water	2N no. eggs in irradiated water
Cleavage time: (tested after 25 min. in solution)	47 min.	46 min.	135 min.	103 min.	46 min.	45 min.

<i>Experiment 24</i>	2N no. eggs in control sea water	N no. eggs in (1:1,000,000) H <sub>2</sub> O <sub>2</sub>	2N no. eggs in (1:1,000,000) H <sub>2</sub> O <sub>2</sub>	N no. eggs in irradiated water	2N no. eggs in irradiated water
Cleavage time: (tested after 50 min. in solution.)	46 min.	53 min.	45 min.	52 min.	45 min.
% Fertilization:	100 fert.	100 fert.	100 fert.	56 fert.	100 fert.

## DISCUSSION

These results are in agreement with the excellent work of Taylor, Thomas, and Brown (1933) and indicate that when test objects are heavily irradiated (around 100,000 r) in water, the possibility of an indirect effect through production of hydrogen peroxide must be taken into account. Conditions of the medium which have been found to affect the amount of hydrogen peroxide remaining in an irradiated solution are (1) the oxygen content, (2) the pH, (3) temperature, (4) radiation intensity (Fricke, 1934a, 1934b, 1935; Risse, 1930), (5) presence of substances which remove the H<sub>2</sub>O<sub>2</sub> (Taylor, Thomas, and Brown, 1933), and (6) the amount of energy absorbed (Bonet-Maurey and Frilley, 1944).

Interference of radiation-produced hydrogen peroxide was reduced to a negligible amount in the earlier investigation (Evans et al., 1942a) by using such dilute suspensions of sperm that they were inactivated by doses (from 2,000 r to 10,000 r) too low to produce detectable amounts of hydrogen peroxide. Also, as hydrogen

peroxide in low concentrations acted only after a time, its toxicity was avoided by removing the sperm immediately from the irradiated water. Taylor, Thomas, and Brown (1933) reduced the toxicity by adding substances possessing catalase activity during the irradiation. In the present investigation, results of attempts to reduce the hydrogen peroxide content by presence of catalase extract during irradiation were puzzling in that the medium possessed toxicity, and it was not removed by active catalase. This finding does not necessarily indicate that irradiation of catalase produces toxic by-products, as many other substances were probably present. However, irradiation of water containing living sperm did not result in toxicity of the medium (Experiment 21-F, Table IV). Other data have been obtained<sup>8</sup> which indicate that catalase *in vivo* is not as easily destroyed by radiation as the catalase extract *in vitro*. Still another possibility is that so many activated water molecules transferred their energy to living sperm that only few remained to produce  $H_2O_2$ . Presence of 0.1 per cent albumin did not appreciably affect the production of hydrogen peroxide (Table IV), but possible competing action at higher concentrations was not studied. Catalase extract added to dilute sperm suspensions afforded some protection against the effect of the activated water molecule as studied in the earlier investigation (Evans et al., 1942a). This protection during irradiation was apparently a competing action rather than due to removal of hydrogen peroxide as the active enzyme was no more effective than heat-inactivated catalase extract. The effectiveness of a protecting substance has been indicated by a ratio of the "Median Effect Dose" for sperm in water plus substance over that of sperm in sea water alone. If active catalase is designated as *A* and inactive catalase as *B*, the ratios were as follows: Experiment 1, *A* = 1.12 and *B* = 1.19; Experiment 2, *A* = 1.6 and *B* = 1.53; Experiment 3, *A* = 2.7 and *B* = 2.6.

In the previous investigation (Evans et al., 1942a) it was concluded that cleavage delay was caused directly by the radiation, whereas fertilizing capacity (and viability) was reduced indirectly through action of an intermediary (activated water molecule). It is interesting to consider that in the present investigation it was demonstrated that, by producing hydrogen peroxide, the activated molecule could indirectly affect cell division as well as cell viability. The direct effect of the radiation on cleavage delay is considered as the loss of some factors from the nucleus which is replaced in time in irradiated eggs, but not in irradiated sperm through metabolic activity (Henshaw, 1940, I-V; Lea, 1946). Recent discussions of radiation inhibition of cell division (Hevesy, 1945; Mitchell, 1943; and Lea, 1946) suggest the possibility that the effect is due to disturbances in nucleic acid and carbohydrate metabolism by means of enzymatic inactivation. It is generally accepted that hydrogen peroxide poisons many enzymes and that the presence of catalase in cells removes the hydrogen peroxide as it is formed in metabolism. The writer has recently investigated the possible relation between radiation destruction of catalase and radiation production of cleavage delay, but the results indicated that the effects were not necessarily correlated.<sup>8</sup> However, one may still consider that, if both  $H_2O_2$  and radiation retard the functioning of systems which regulate cell division rate, their mode of action may be similar (i.e., oxidation) in producing this effect. The question as to how the active agent in irradiated water (hydrogen peroxide) might have exerted its effect of reducing survival time leads one to consider factors which affect longevity of untreated sperm. This subject has been studied recently by Hayashi (1945-1946) who concluded that a factor prolonging

the duration of fertilizing capacity of Arbacia sperm was adsorbed on the cell surface, and subsequently was lost into the surrounding medium. Seminal fluid contained a protein which, by its action on the surface of the sperm, may maintain fertilizing capacity and respiratory rate. Some findings in the present investigation suggest that the specific protein complex on the surface of the sperm is present even when removed from the seminal fluid by washing and centrifuging, and that its destruction can be retarded by non-specific proteins. This is indicated by the observation that sperm, after removal of seminal fluid, could be kept alive and fertile for extended periods of time (even for days) as long as they were closely packed. In sea water the factor was lost and disappeared more rapidly the more dilute the suspension. It may be noted in the earlier report (and in Table IV, Experiment 129 of this report) that addition of a non-specific protein (egg albumin) increased the survival time of sperm in sea water. Two additional experiments will be cited. In one experiment 1:1,000 sperm in 0.1 per cent albumin retained their fertilizing capacity 2.6 times as long as the same concentration of sperm in sea water alone. Sperm in sea water plus albumin, in the other case, remained fertile 14 times longer than sperm in sea water without the albumin (this is the same value as obtained in Experiment 129, Table IV). Sperm in water plus catalase extract remained fertile 1.8 times as long as sperm in water alone. This prolonging of viability was not due to the enzyme activity as heated catalase extract had exactly the same effect (80 per cent increase in survival time). It may be of interest to mention in this connection the finding of Saul and Nelson (1935) and Adams and Nelson (1938) that when purified preparations of invertase or tyrosinase are highly diluted they lose activity. If serum albumin, etc., is added during the dilution, or immediately thereafter, this loss in activity is prevented and activity measurements run proportional to the dilution factor. Hayashi (1946) considers that the fall in activity of Arbacia sperm in sea water is due to destruction of a system involving the cell surface, to auto-intoxication and probable depletion of fuel. One type of auto-intoxication could be auto-oxidation (production of  $H_2O_2$ ). If this is true then the irradiated water may act by removing catalase from the surface which would, in turn, allow accumulation of  $H_2O_2$  formed during respiratory metabolism. Action of the activated molecule could be due to direct oxidation of materials at the cell surface or to destruction of protective catalase.

#### SUMMARY

Heavily irradiated water (over 100,000 r) was found to have deleterious effects on sperm placed therein. These effects were reduction of survival time and delay in first cleavage when such treated sperm were used to inseminate fertile eggs. The injury became more pronounced the greater the irradiation dose and the longer the sperm remained in the irradiated water.

The chief, if not the only, agent responsible for these effects of irradiated water was hydrogen peroxide. This was shown by chemical test, by similarity of its action with that of hydrogen peroxide, and by removing the toxicity with catalase extract.

The effects of hydrogen peroxide on fertility and on subsequent cleavage time are discussed regarding possible interpretations of similar reactions of the sperm to more direct effects of roentgen radiation.

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# DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER.\* VI. THE EFFECT OF ENZYME POISONS

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When organic matter, in the form of natural marine plankton, is allowed to decompose in sea water in the dark under laboratory conditions ammonia is first found in the water, followed by nitrite, and later by nitrate, in amounts corresponding to the nitrogen which disappears from the suspended particulate matter. When, in the last stage, the water is inoculated with fresh diatoms a new flowering takes place in the light, with the disappearance of nitrate from the water. In darkness, this new organic matter again decomposes and the cycle repeats itself. Many of the features of this cycle, and the effects of such variables as time, temperature, source of water and organic matter, etc., we have reported in previous papers (1937-1942).

To renew these studies, after an enforced delay of three years, we chose to investigate the effects of certain enzyme poisons on the several stages of the decomposition and regeneration cycle. The plan was to start decomposition "cultures" consisting of natural sea water and marine phytoplankton material and to follow the ammonia and nitrite in the water, in control portions as well as in portions to which enzyme inhibitors were added at various stages in the cycle.

Since the effects to be observed were at most semi-quantitative it was possible to simplify the analytical techniques to reduce the laborious character of the ammonia analyses and to eliminate the determination of nitrate and particulate nitrogen. The distillation procedure for ammonia was identical with that previously described, but after collecting the distillate the ammonia in it was finally determined by Nesslerization rather than by titration with hypobromite. While not as accurate, this procedure was sufficient for the purpose and more rapid. Nitrite was determined by the same colorimetric method as before, with the use of the Klett-Summerson photoelectric colorimeter in the later stages of the work.

## EXPERIMENTAL

Four decomposition "cultures" were prepared (Nos. 101, 103, 104, and 105) from water obtained in Vineyard Sound near Woods Hole. Phytoplankton was collected in a double net, so that the larger organisms were eliminated. The tow consisted principally of *Rhizosolenia*. Varying, undetermined quantities of this material were added to the water in 5-gallon carboys, No. 104 containing the largest amount and No. 103 the least. Smaller portions (about 2 liters) were removed to separate containers and the inhibiting substances added in fixed concentrations. These were allowed to decompose in the dark, at laboratory temperature, simultaneously with the main "control" portion of each culture. At intervals, determina-

\* Contribution No. 371 from the Woods Hole Oceanographic Institution.

tions of ammonia and nitrite were made and at certain times portions were again removed from the main culture and inhibitors were added.

The enzyme poisons used were: KCN, in concentrations from 0.0001 to 0.01 M; NaF, 0.01 and 0.03 M;  $\text{As}_2\text{O}_3$ , 0.01 M; sodium iodoacetate, 0.01 M; ethyl, propyl and *n*-butyl carbamates, 0.2 M. These were rendered neutral by addition of acid or alkali as required and were added at various stages in the cycle: at the beginning; when the ammonia had reached a maximum; and when the ammonia had been entirely replaced by nitrite.

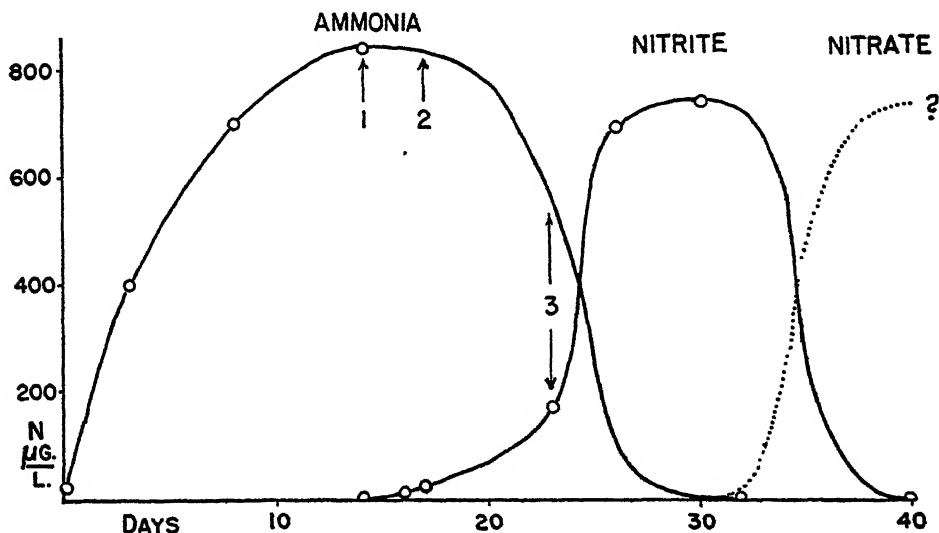


FIGURE 1. Series No. 104. Formation of ammonia, nitrite (and nitrate by inference) during decomposition in the dark. Source of organic matter, principally *Rhizosolenia* suspended in water from Vineyard Sound. Time in days, nitrogen in micrograms per liter. At points indicated by 1, 2, and 3 portions of this control culture were removed and to them were added various enzyme poisons, as described in the text.

Figure 1 shows the course of the cycle in Series 104. In the control, ammonia reached its maximum after about 15 days, at which time nitrite was first detected. The latter reached its maximum about 15 days thereafter, and in another 10 days—or 40 days from the start of the decomposition—it had again disappeared entirely. We know from previous experience that complete oxidation to nitrate has taken place at this time. At the beginning of the experiment five 2-liter portions were removed, to which were added respectively: A, KCN, 0.01 M; B, KCN, 0.001 M; C, KCN, 0.0001 M; D, NaF, 0.03 M; E,  $\text{As}_2\text{O}_3$ , 0.01 M. At the point marked 1 in Figure 1, at 14 days, portions were removed to which were added: F, KCN, 0.01 M; G, KCN, 0.001 M; H,  $\text{As}_2\text{O}_3$ , 0.01 M. At point 2, at 17 days, similar sub-cultures were prepared containing: J, ethyl carbamate, 0.2 M; K, propyl carbamate, 0.2 M; L, sodium iodoacetate, 0.01 M. At point 3, sub-cultures were prepared containing: M, KCN, 0.001 M; N, KCN, 0.0001 M.

Series 101 and 103 were carried out in the same manner, except for variation in the times at which the inhibitors were added. Series 105 was carried through the

first half of the cycle only, to learn the effects of the carbamates and of iodoacetate on the liberation of ammonia.

## RESULTS

The results and conclusions are based upon observation of the relative rates of appearance and disappearance of ammonia and nitrite in the presence and absence of inhibitors. The graphic details of these experiments need not be reproduced, but the following observations were made as to the specific effects of the various poisons.

### KCN

In no case did significant amounts of nitrite appear in any culture to which KCN was added, even in concentrations as low as 0.0001 M. When KCN was added, even in the lowest concentration, to cultures in which nitrite had already appeared, the latter did not increase in amount, showing that the oxidation of either ammonia or nitrite was inhibited. In 0.0001 M concentration it had no effect upon the formation of ammonia, but in concentration of 0.001 or 0.01 M it greatly retarded the rate. In Series 104, for example, in which ammonia had disappeared in about 30 days in the control culture, less than half the maximal amount had appeared in 46 days in the presence of 0.01 M KCN.

### Carbamates (*ethyl, propyl, n-butyl*)

When carbamates were added in 0.2 M concentration to cultures already containing ammonia no nitrite formed. If nitrite had already appeared before carbamate was added it did not further increase. Carbamate therefore appears to inhibit the oxidation of both ammonia and nitrite. It was not possible to determine the effect of carbamate upon the formation of ammonia since it interfered with the analysis, decomposing during the distillation and also rendering the Nessler reagent inactive.

### Iodoacetate

Iodoacetate inhibited both the formation and further oxidation of nitrite, exactly as cyanide and carbamate. A single experiment indicated that it permitted the formation of ammonia, but somewhat more slowly than in its absence.

### NaF

In a concentration of 0.03 M sodium fluoride did not interfere with ammonia formation, but in this concentration, as well as in 0.01 M, it prevented oxidation to nitrite. It should be mentioned, however, that these concentrations produce a copious precipitate of  $\text{CaF}_2$ , so that the resulting concentration of fluoride ion is considerably smaller. The solid precipitate itself may also be a disturbing factor.

### $\text{As}_2\text{O}_3$

Nitrite did not appear when  $\text{As}_2\text{O}_3$  was present. Its effect upon ammonia formation was inconclusive; in one case ammonia appeared but in another it did not. It proved difficult to keep the solution neutral, however, and in at least one case a strong acid condition developed which was not detected until the close of the experiment.

## DISCUSSION

Microorganisms may produce ammonia through various chemical mechanisms: hydrolytic deamination, reductive deamination and oxidative deamination (Waksman, 1932). Depending upon conditions (for example, aerobic or anaerobic surroundings) and the species of microorganisms present one or the other process will predominate. It is consequently not surprising that variations in the response to inhibitors should occur, since the various mechanisms will involve different enzyme systems. The different response to  $\text{As}_2\text{O}_3$  observed in two series of the present study can be easily understood on the assumption of the presence of different microbial floras. The non-interference of this poison with ammonia production in one of the series is not surprising; it has been found that the deamination of amino acids by kidney slices proceeds also in the presence of arsenite (Krebs, 1933). There is also a parallel to the ineffectiveness of iodoacetate; Geimann (1936) reports that it does not suppress the ammonia formation within the tissues of the medusa *Aurelia*.

The fact that cyanide retards, but does not completely suppress, ammonia formation is of interest. It is known that all cases of oxidative bacterial deaminations so far studied are inhibited by 0.005 M KCN (Stephenson, 1939). Our observations may therefore be taken to indicate that under normal conditions oxidative deaminations occur and predominate in decomposing plankton samples. They are probably catalyzed by heavy metals. In our cyanide-inhibited samples ammonia formation would then be due to one of the other mechanisms. We cannot decide at the present time whether the slower rate of ammonia formation is due to a weakly developed alternate mechanism in the same bacteria whose oxidative deamination mechanism has been inhibited. It is perhaps just as likely that such organisms have been more or less completely eliminated and that a longer time is required to build up a bacterial population capable of liberating ammonia from protein or its higher decomposition products by a mechanism that is not sensitive to cyanide.

While the production of ammonia is common to many groups of bacteria the formation of nitrite and nitrate is restricted to specialized types. Meyerhof (1916a and b; 1917) studied the metabolism of soil nitrifiers belonging to the two groups involved. He found that both are sensitive to urethanes and that the nitrate formation is also inhibited by cyanide, but he did not study the effect of cyanide upon the nitrite-forming flora. The present study confirms these observations from the corresponding floras occurring in the sea and also establishes the cyanide sensitivity of the ammonia-oxidizing flora.

The action of cyanide can very likely be ascribed to an enzymatic inhibition. This is indicated by the observation that even very small concentrations of this poison can suddenly interrupt both the nitrite and nitrate formation; i.e., inhibition takes place even when the floras have already been built up. One is not dealing, therefore, merely with suppression of the development of the necessary number of microorganisms.

It seems likely that the formation of both nitrite and nitrate is catalyzed by heavy metals. It is possible that the cytochrome system is involved. This seems to follow from the fluoride inhibition. It has been shown in a variety of biological materials that fluoride attacks the cytochrome system; the actual point of attack seems to be cytochrome *c*, rather than the cytochrome oxidase itself (Borei, 1945). It would



be of interest to study more fully the possible role of the cytochromes during nitrite and nitrate formation.

Iodoacetate and arsenite, in the concentrations employed, are primarily dehydrogenase inhibitors. The inhibition of nitrite and nitrate formation by these poisons seems to indicate that enzymes of this type are also involved. It is, of course, a well established fact that both oxidases and dehydrogenases are frequently linked in one chain and that both are necessary to complete an oxidative process.

The inhibition observed in the presence of carbamates would under ordinary circumstances strengthen the assumption of the participation of dehydrogenases, since these narcotics are usually considered as dehydrogenase inhibitors. However, in the present case one must be cautious in interpreting the results. It is a well established fact that many organic compounds are definitely toxic to nitrifiers, especially in high concentrations (Meyerhof, 1916b; Fred. and Davenport, 1921). For the present, therefore, we cannot rule out the possibility of a non-specific action of the carbamates.

A practical outcome of the present study is the suggestion that cyanide, in exceedingly low concentrations, may be an effective preservative for sea water before analysis for nitrite and nitrate. Various points, however, such as the minimum concentration of cyanide, the time limits of protection, etc., must be studied before it can be recommended for routine use.

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THE BEHAVIOR AND METAMORPHOSIS OF THE LARVA OF  
BUGULA NERITINA (LINNAEUS): EXPERIMENTAL MODI-  
FICATION OF THE LENGTH OF THE FREE-SWIM-  
MING PERIOD AND THE RESPONSES OF THE  
LARVAE TO LIGHT AND GRAVITY

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McDougall (1943) noted that the larvae of *Bugula neritina*, when liberated in finger-bowls containing sea water at room temperature (25–30° C.), swam without exception towards the source of illumination and within an hour the majority became attached to the surface film or to the sides of the container. The larvae remained positively phototropic throughout their pelagic stage. While experimenting with light boxes containing chambers arranged at the same horizontal level but receiving different amounts of illumination, however, he found 1,533 settings in the two chambers receiving the least amount of illumination, but only half as many in the two most brightly lighted ones. Furthermore, on tiles submerged in Beaufort Channel the most intense vertical distribution of attached larvae was very near the bottom at a depth of 79–104 inches. Their numbers gradually decreased up to a point 6 inches below mean low watermark; presumably the inability of *Bugula* colonies to tolerate exposure to air for more than a few minutes accounts for their absence above this level. These observations indicate that larvae in their natural environment become photonegative and geopositive some time before attachment. McDougall (1943) called attention to the marked contrast between the behavior of the larvae under laboratory conditions and their apparent behavior in nature.

The studies reported in this paper were undertaken to determine what environmental factors may be responsible for the observed distribution of adult colonies at Beaufort, North Carolina. Incidentally they led to an investigation of the mechanisms involved in causing metamorphosis.

The general literature on the development and metamorphosis of the Bryozoa is fairly extensive. A monograph by Barrois (1877), reviewing the behavior, development and metamorphosis of representative types of both endoprocts and ectoprocts, is the most comprehensive single treatise in the literature. Other important contributions have been made by: Schneider (1869), Claparède (1870), Nitsche (1870), Metschnikoff (1871), Allmann (1872), Salensky (1874), Schmidt (1876), Hatschek (1877), Joliet (1877), Repiachoff (1877), Barrois (1879; 1882; 1886), Hinks (1880), Harmer (1885; 1887; 1903; 1931), Ostroumoff (1885; 1886), Vigelius (1886), Pergens (1889), Prouho (1890; 1892), Seeliger (1890; 1906), Calvét (1900), Kupelwieser (1905), Marcus (1921; 1926a, b, c), Waters (1925), O'Donoghue (1926), B. H. Grave (1930), and Rogick (1939).

Although a considerable amount of experimental work has been done during the past fifteen years on the behavior and metamorphosis of larval ascidians and a few

other sessile organisms, only a few observations have been recorded for the Bryozoa. The swimming movements of the larvae and their reactions to light have been described briefly in the earlier literature; but attempts to modify their behavior under controlled conditions have been confined largely to those of Marcus (1926a, c) and B. H. Grave (1930). The latter dealt exclusively with experimental modifications of the reactions of the larvae to light. The former, by employing vertical temperature gradients, demonstrated the existence of a fright-reaction and a lethal temperature zone at 38° C. for the larvae of the fresh-water species, *Plumatella fungosa*. He also briefly recorded the failure of these larvae to respond to an increasing oxygen-gradient and their lack of conformity to the generally accepted rules for determining the effects of viscosity and temperature on the speed of swimming movements.

McDougall (1943) discussed the breeding period, growth-rates and seasonal distribution of colonies of *Bugula neritina* in the Beaufort region and, after briefly referring to the behavior of the larvae, emphasized the need for further research. Since the embryology of this species has not yet been reported, a brief description of the larva, an account of the events that take place during metamorphosis and the duration of its various phases are presented. A review of the literature shows that the earlier embryologists only vaguely described the length of the post-fixational sequences of other species of Bryozoa. Among more recent writers, B. H. Grave (1930) has given an excellent description of the larvae of *B. flabellata* and *B. turrita* from the Woods Hole region and has recorded the growth rates during the later embryological stages, but his account of the early phases of metamorphosis is somewhat brief.

#### DISTRIBUTION OF BUGULA NERITINA

This semi-tropical species is abundant in the Dry Tortugas, Florida (Osburn, 1914), in Japan (Miyazaki, 1938), in Hawaii (Edmondson, 1944) and on the coast of California (Robertson, 1905). Canu and Bassler (1929) found it in the Philippines; Verrill and Smith (1874) have recorded it for the Bermuda Islands, Calv  t (1900) for the southern coast of France and Osburn (1927) for Cura  ao. Although it apparently does not range farther north on the Atlantic Coast than Beaufort, North Carolina, it is replaced along the northern half of the East Coast by a closely related species, *Bugula turrita*, which can be distinguished from it by the presence of avicularia.

The adult organisms are purplish-brown, branching colonies, which may reach a length of 10-12 cm. McDougall (1943) found that the vertical distribution of these colonies was most dense at a distance ranging from 6 inches to 3 feet below mean low water. Colonies near the surface were longer than those nearer the bottom and growth was more luxuriant. The distribution of adult colonies, therefore, differed considerably from that of the larvae. The writer found the bottom of a large raft to be the most suitable place for collecting adult specimens, which grew luxuriantly in close association with the ascidian, *Perophora viridis*. *Pennaria*, which is frequently associated with *B. neritina*, grew abundantly along the sides of the raft, but was almost entirely absent from the bottom. Light seems to be a controlling factor in causing the former organisms to attach and grow in deeply shaded regions and the latter in places exposed to bright sunlight. Along the western coast of North America, *B. neritina* becomes the dominant pile-dweller

during November, when it replaces the formerly predominant algal community, with which it is closely associated (Scheer, 1945).

Since *B. neritina* grew abundantly near the laboratory on rafts and pilings within arm's length of the surface, it was surprising to find a much altered situation less than a mile up the mouth of the Newport River. Here Bryozoa could not be found at all on pilings within three feet of the surface. Scrapings from regions lower down failed to bring up any specimens. A few sparsely growing colonies were found near the bottom of submerged objects that could be raised to the surface from a depth of 6 or 8 feet. Since samples of the water showed a surprising decline in salinity within a range of half a mile, it was considered probable that the salt content might be a controlling factor in bringing about the observed horizontal distribution in this region.

### MATERIALS AND METHODS

Since the histological details of the development of several species of Bryozoa are well-known, the writer used only living material for the embryological observations and the experimental work. Through the facilities offered by the U. S. Bureau of Fisheries, Beaufort, North Carolina, the larvae for these studies were obtained from sexually mature colonies placed in finger-bowls and kept in running water. These colonies usually yielded a considerable number of larvae each day for a period of ten days to two weeks. In some instances, however, larvae were produced in enormous numbers for five or six days; but at the end of that time they were no longer shed, and the colonies had to be replaced with fresh ones. During the summers of both 1944 and 1945 it was observed that there were periods lasting about a week when larvae were almost unobtainable in large numbers, even when fresh colonies had been gathered the night before. These periods occurred at approximately the same time each year, towards the end of the first week in August. Since there seemed to be no unusual variations in temperature or sunshine, the cause of this phenomenon was not determined.

Although it would be preferable, indeed, to use only larvae from a single colony for each set of experiments, nevertheless the small number that would be obtainable by such a procedure practically precludes the attainment of such a desideratum. Some of the variations in the behavior of the larvae under similar experimental conditions may be attributable, perhaps, to differences in genetic strains. It seems more likely, however, that the length of time that the larvae have remained in the ovicells before being shed is a more important factor in introducing seemingly fortuitous variations in the length of the free-swimming period. The larvae, as a rule, show a remarkable uniformity in their behavior under similar environmental conditions; genetic variations seem to play only a minor role in determining the length of the free-swimming period.

- For observations on the responses of the larvae to light and gravity at reduced temperatures, stender dishes 5 cm. in diameter and 3 cm. deep were used exclusively during the first summer. During the second summer, homeopathic vials 8 cm. long and 1.5 cm. in diameter were used for these observations and for those on the effects of salinity. For most of the light experiments, vials 16 cm. long and 1.8 cm. in diameter were employed. These were scrubbed carefully with soap and water and then rinsed thoroughly with sea water. A light-box, 8 × 8 × 30 cm., was

constructed so that light could enter only one end of a vial placed within it. Well-slides were used for microscopic work.

Shortly before the experiment was to begin, finger-bowls containing adult colonies were placed near a window that admitted diffuse daylight to the laboratory. When larvae were liberated, they swam immediately towards the side of the dish nearer the window and collected there in swarms just beneath the surface film. They could then be pipetted to the various experimental vials, which were numbered so that a record of the time of placement could be kept. Since the liberation of larvae is photoperiodic, generally beginning 30–40 minutes after the parent colonies are exposed to light, active specimens could be obtained throughout the day, if the containers were placed in a darkroom on the preceding evening and removed to a window a short time before the larvae were needed. Although active swimmers could be obtained as late as 3:30 P.M. (E.S.T.) by this method, the number of larvae shed in the afternoon was generally small in comparison with the number liberated between 7 A.M. and noon. Since the darkroom was not equipped with a system of running sea water, the food supply of the adult colonies may have been inadequate under these conditions for the proper development or liberation of larvae. The finger-bowls were generally kept in the aquarium, which was situated in a fairly well-shaded spot; only on days when the room was flooded with light from an early hour were the larvae shed in large numbers before the experiment began. Observations were usually made between 7 A.M. and noon, although in some cases they had to be carried out until 6 P.M. and in one case until 10 P.M. Larvae were most abundant during the first two hours after exposure of the parental colonies to light.

#### OBSERVATIONAL SECTION

##### *Structure of the larva*

The larva of *B. neritina* resembles other larvae of the same genus in its structural features and its general contour. It may be described as pyriform rather than peach-shaped, however, with average dimensions  $0.20\text{--}0.22 \times 0.27\text{--}0.30$  mm., although smaller specimens were observed at times. At the narrower end of the body the convex apical organ, the "calotte" of Barrois (1879), is clearly set off from the rest of the larva by a crown of rigid cilia and by a circular groove or collarete, the pallial furrow (Fig. 1). Its coloration is somewhat lighter than the rest of the larva, which looks green by reflected light, but brown by transmitted light because of the numerous pigment granules imbedded in the body. The middle of the apical organ has a darkly pigmented spot whose center shows a tiny clear area that sometimes disappears when the surrounding tissue suddenly contracts (Fig. 4). This spot marks an opening into a shallow cavity in the interior of the larva. The broader end, which corresponds to the oral depression of larvae that have a rudimentary or functional alimentary canal, contains in its center an invagination forming the relatively voluminous internal sac or sucker, the "saugnapi" of Pergens (1889), whose opening to the outside is marked by a ring of black pigment (Fig. 3). The internal sac becomes so distended by a secretion of granular material from its walls that the whole region between the equator and the oral depression bulges like the broader end of a pear. The median furrow, which B. H. Grave (1930) called a lateral groove, is clearly defined by its lighter coloration and by the absence of pigment (Fig. 2). In the median furrow near the equator is a tuft of four, long,

blunt vibratile flagella that beat in unison. This is the "plumet ciliaire" of Barrois (1877). The median furrow and its associated glandular structures constitute the piriform organ. In some specimens there are two, small, darkly pigmented eye-spots near the tuft of flagella and symmetrically placed with reference to the median furrow. They can be seen from the opposite side through the somewhat transparent larva (Fig. 1). On the side of the larva opposite the median furrow are two, prominent, black, diamond-shaped eye-spots lying almost on the equator and about 90° apart. These structures are a constant feature of the larvae and serve as excellent landmarks for their proper orientation during swimming movements. A tiny, white area that appears frequently in the center of each eye-spot, when light falls at the proper angle, is somewhat difficult to interpret; probably it is caused by a concentration of light brought about by a crystalline lens that covers the eye-spots, as Nitsche (1870) has described for another species of *Bugula*. The locomotor cilia, which cover the body except in the region of the apical organ, are more active near the apical organ than on the half of the body containing the oral depression; if stationary larvae are observed, water currents can be seen flowing from the region near the apical organ to the equator and then outwards and back again to complete a circle. Since locomotor cilia of most bryozoan larvae are found only on the enlarged coronal cells that form an elevated ring around the body of the organism, Barrois (1877) has interpreted the presence of cilia on the whole body of *Bugula* larvae as an indication that the corona has spread out enormously in these forms, so as to occupy the whole region between the apical organ and the oral depression. This view is supported by the fact that the corona of *Flustrella hispida* is a single band around the oral pole, whereas it is separated into two distinct bands in the larva of *Cyphonautes compressus* and in other forms having a similar type of larva. It is quite possible, however, to regard the condition in *Bugula* as primitive; condensation of the cilia into bands may not have occurred until a later period of phylogenetic development.

Changes in body contour, accompanied by elongation of the larvae, can be observed frequently. In some cases larvae look lobular rather than pear-shaped, as though they were divided into four lobes by two deep constrictions running at right angles to each other (Fig. 5). This appearance results from a contraction in the region of the equator accompanied by a depression of the apical organ.

#### *Orientation and swimming movements of the larva*

The larvae usually swim with their long axes tilted at an angle of 45° with the vertical, if they are advancing in a definite direction and are not in contact with the bottom of the container or the surface film. Both the apical organ and the tuft of vibratile flagella are in advance and the median furrow is directed downward. In this respect the larvae differ from those of *B. flabellata*, which swim with their long axes directed horizontally, and from the larvae of the Escarina, in which the long axes are vertical (Barrois, 1877; 1886). Spiral movements, however, can be observed frequently. While spiralling, the organism usually has its median furrow on the outside of the spiral. This is probably why B. H. Grave (1930) called it a lateral groove. (He clearly described the function of the flagella and cilia in these movements.) If the larvae are in contact with parts of the container or the surface film, they progress by creeping movements with the median furrow and the vibratile flagella in contact with the surface. Sometimes, while on the bottom, they spin on

their long axes and show in quick succession first the median furrow and then the pigmented eye-spots.

Observations of these diverse methods of locomotion have brought about a certain amount of confusion in the literature on closely related species. Calv  t (1900), for instance, in referring to the larva of *B. sabbatiere* described the half containing the eye-spots as posterior and the other half bearing the median furrow as anterior. B. H. Grave (1930), on the other hand, referred to the apical organ of *B. flabellata* as the anterior end. His orientation is in agreement with that of Barrois (1877). As Grave (1930) noted, it is necessary to distinguish the anterior half of the larva, as determined by its forward motion, from the morphological anterior end; for the mouth, when one is present in bryozoan larvae, is opposite the apical organ. Barrois (1877) cited similar cases showing the different interpretations of various observers regarding the orientation of the cyphonautes larva of *Membranipora pilosa* (*Electra pilosa*) (p. 232). It seems worth noting that the sensory structures, the apical and piriform organs, are in advance as the larva moves forward and first come in contact with the environment. According to Prouho (1890) the apical organ of *Flustrella hispida* is connected to the piriform organ and also to the cells of the corona by nervous tissue. There can no longer be any doubt about the sensory nature of either the apical or piriform organs.

During the early part of the larval period, relatively long distances are covered by a spiralling motion. Finally there is a slowing down of movement, accompanied by a change in the method of progression. Spiralling ceases, and the larvae now swim in circular planes parallel to the substrate; the radii of the concentric circles formed by the larvae decrease in length as the onset of metamorphosis approaches. Eventually they slow down enough to be kept within the low-power field of the microscope. While swimming in circles, larvae often behaved as if a very fine, sticky thread trailed beneath the piriform organ, which was downward at the time. Although the thread itself could not be seen, several larvae were observed to pull debris, with considerable difficulty, at a distance of several millimeters; they could be pushed and pulled with a probe at a short distance from their bodies.

Before fixation, larvae alight on a suitable substrate and begin to rotate counter-clockwise on an axis running from a point midway between the eye-spots to the opposite side, where the piriform organ touches the substrate. This continues for 5

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#### PLATE I

FIGURE 1. Profile view of the larva showing pigmented eye-spots. 1. Convex apical organ, 2. Crown of rigid cilia, 3. Collarete or pallial furrow, 4. Eye-spots on the opposite side, seen through the somewhat transparent larva, 5. Ciliated corona, 6. Eye-spot, 7. Pigmented band.

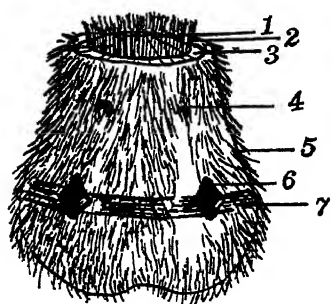
FIGURE 2. Profile view showing the median furrow and associated structures (the piriform organ). 1. Part of the glandular system, 2. Eye-spot, 3. Tuft of vibratile flagella, 4. Median furrow, 5. Oral depression.

FIGURE 3. A three-quarters view showing the position of the long axis during spiral movements. 1. Opening to the internal sac, 2. Oral depression.

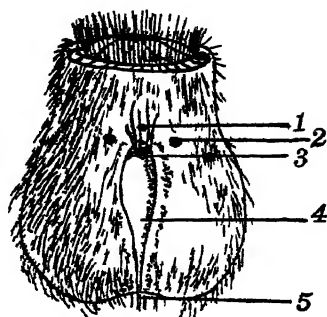
FIGURE 4. View showing the apical organ. 1. Opening in the apical organ, 2. Crown of rigid cilia, 3. Pallial furrow.

FIGURE 5. View showing the larva compressed in the region of the equator with the apical organ retracted. 1. Retracted apical organ.

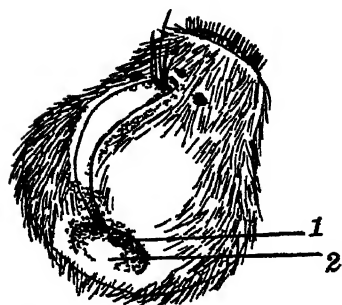
FIGURE 6. Profile view, 15 seconds after fixation, showing characteristic hour-glass appearance. 1. Pallial furrow, 2. Vibratile flagella, 3. Ejected holdfast.



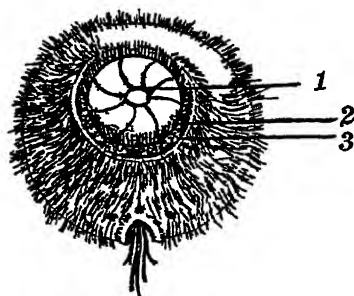
*Fig. 1*



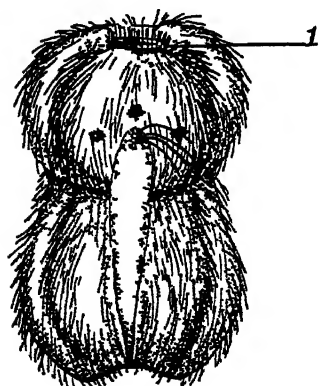
*Fig. 2*



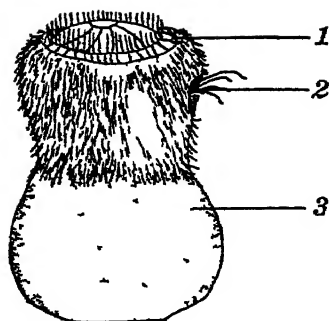
*Fig. 3*



*Fig. 4*



*Fig. 5*



*Fig. 6*



or 10 minutes. Rotation gradually decreases in speed and finally stops just before attachment. The flagella of the vibratile plume enable the larvae to choose places suitable for fixation. Light and shadow undoubtedly play an important role in the selection of a favorable spot.

### *Metamorphosis*

Just before fixation the larva actually appears to be anchored to the point of attachment by an invisible thread-like substance. If it is going to attach to the bottom of a well-slide, it alights with the eye-spots uppermost and the median furrow along the substrate. While it revolves slowly, always in a counterclockwise direction, it changes its shape, so that it looks like an oblate spheroid, bulging at the equator and flattened in the region of the apical organ and oral depression. Perhaps these changes in body contour indicate the establishment of a new polarity just before fixation, since Child (1925) found that a reversal of polarity occurs in hydrozoan larvae immediately before attachment.

Before setting, the vibratile flagella are very active, feeling the surface of attachment, while the larva revolves slowly for 3 or 4 minutes. At the moment of fixation the median furrow grasps the substrate by means of its ridges, which are both muscular and glandular. Within a second or two the larva elongates, so as to double the length of the long axis of its body. Then very suddenly the internal sac, containing a white, translucent, slightly granular, jelly-like material, is everted and forms a light colored, rounded mass beneath the organism, which is almost as large as the larva itself.

Simultaneously with the eversion of the internal sac, the median furrow releases its grip on the substrate and the larva rotates, so as to change the original long axis of the organism from a horizontal to a nearly vertical position. The apical organ, therefore, is brought upwards, but in such a manner that it faces away from the source of illumination at an angle of 15 or 20° from the vertical. As the larva rotates, it orients itself by squirming movements, until the eye-spots are located on the lighted side. The writer did not observe that the apical organ in this species points towards the source of illumination at the time when metamorphosis commences, as Grave (1930) has described for *B. flabellata*. After fixation the aboral

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### PLATE II

FIGURE 7. "Umbrella stage" (semi-diagrammatic optical section). 1. Epithelium from the pallial furrow, 2. Holdfast.

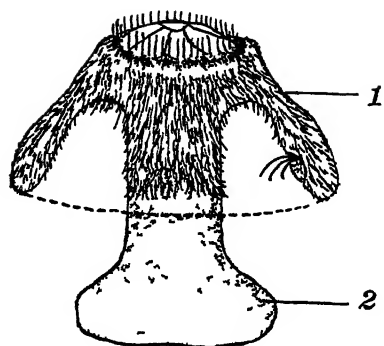
FIGURE 8. Diagram showing epithelium from the pallial furrow forming a firm union with the holdfast. 1. Epithelium from the pallial furrow, 2. Reflected original larval covering.

FIGURE 9. The fixed larva viewed from above, 13 minutes after fixation. 1. Ringed cavity surrounded by degenerating ciliated larval covering (the corona), 2. Ciliated covering in the process of degeneration, 3. Transparent anhiste zone (the holdfast), 4. Eye-spots seen through the new larval covering (epithelium from the pallial furrow).

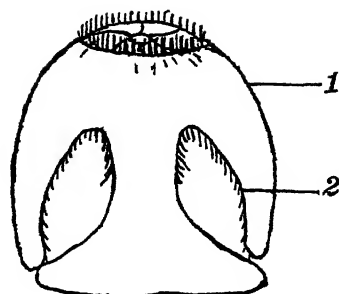
FIGURE 10. The fixed larva, viewed from above, 18 minutes after fixation. The horse-shoe shaped mass has increased in diameter.

FIGURE 11. The cystide, nine hours after fixation, showing a cut in the region where the piriform organ was situated. The rudiment of the polypide can be seen in the center.

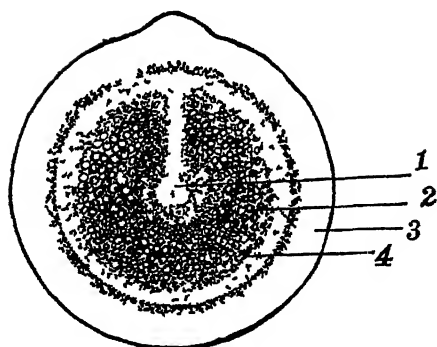
FIGURE 12. The cystide somewhat older than that shown in Figure 11 (about 12 hours after fixation). The rudiment of the polypide is already well-formed. 1. Granular material being extruded into the surrounding water, 2. Break in the outer cystide wall, 3. Rudiment of the polypide.



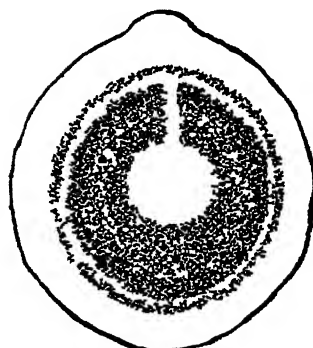
*Fig. 7*



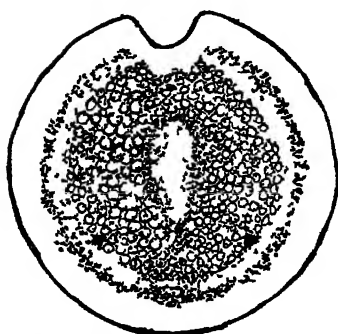
*Fig. 8*  
(diagrammatic)



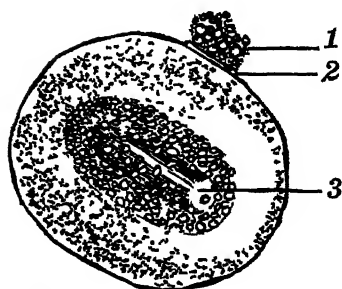
*Fig. 9*



*Fig. 10*



*Fig. 11*



*Fig. 12*

end of the attached larva and later the apical part of the first zoöid are oriented so as to point away from the direction of the incoming light.

With the eversion of the internal sac, which formerly distended the oral hemisphere of the unmetamorphosed larva, the central portion of the body collapses somewhat and forms a narrow column supporting the apical organ on top and connecting it to the upper part of the evaginated internal sac beneath. Thus the central region of the organism becomes constricted and the larva assumes a characteristic hour-glass appearance (Fig. 6). A cavity is formed in the region formerly occupied by the internal sac. This hour-glass appearance lasts only 3 or 4 minutes, while the larva orients itself to light. If the slide is subsequently turned through an angle of  $180^\circ$ , no further orientation will take place; the eye-spots will remain on the side opposite the source of illumination.

Then very suddenly the apical epithelium unfolds from the pallial furrow, where it was tucked-in to form the collarette, and extends outward and downward, carrying with it the upper border of the ciliated larval covering as it migrates towards the adhesive plate (Fig. 7). This is the "umbrella stage" of Barrois (1886). The ciliated covering of the larva thus becomes reflected outward and downward, until it finally becomes completely enclosed by the apical epithelium (Fig. 8). The cells of the latter become stretched and flattened, so that their resultant transparency allows prominent landmarks like the pigmented eye-spots to be seen beneath it. The latter are carried downward during this process, until they finally rest just above the everted internal sac, where they form prominent bulges; they remain visible for 15 or 20 minutes after fixation (Fig. 9). Then with a sudden contraction the apical organ is pulled down towards the everted internal sac, which becomes flattened to form an adhesive plate. The larva now looks like a rounded cup inverted over a plate.

Just after the aboral organ is pulled down towards the adhesive plate, the fixation substance seems to grow larger in diameter, when viewed from the apical pole. This is probably caused by a flattening-out of the adhesive material and by a simultaneous constriction of the central, ciliated, papilla-like structure, which forms a ring of small diameter. At this time the opaque parts of the organism, the ciliated former outer covering and the apical organ, measure 0.14–0.15 mm. in diameter. Then the size of the adhesive plate seems to decrease again and at the same time the central ciliated mass widens, so that the clear area in the center becomes larger (Fig. 10). This appearance of a decrease in the diameter of the adhesive material can be attributed to the beginning of a union of its outer margin with the lower border of the reflected epithelium from the pallial furrow, which forms a new covering for the organism. The adhesive apparatus itself consists of a lower layer of transparent material, probably a cement-like adhesive substance formerly contained in the internal sac, and a smaller layer on top; the latter, which is somewhat pigmented, is the border of the internal sac.

The lower border of the epithelium from the pallial furrow does not form a firm union with the adhesive plate until 20 or 30 minutes after fixation. The cilia, at the margin of the former larval covering and the epithelium from the pallial furrow which covers it, may be seen beating for as long as 10 or 15 minutes after attachment. The vibratile flagella continue to move rapidly at first and then spasmodically for 5 or 10 minutes longer, until the cells to which they are attached have been carried well into the interior. Before these cells have migrated to a position nearer

the center of the larva, the flagella can be seen sticking out on top of the adhesive plate, which forms a prominent bulge in this region (Fig. 9). In many instances granular material was observed to be extruded into the surrounding water, as if the adhesive plate were ruptured by the movement of the flagella. Finally, they retreat completely within the new outer covering.

As a result of all these changes, the vibratile flagella and the associated piriform organ, as well as the whole ciliated original larval covering, have become enclosed by a new outer covering formed by epithelium from the pallial furrow. At this time the larva looks like a sac, closed on the bottom by the adhesive plate; its top and sides are formed by the apical organ and by the epithelium from the pallial furrow respectively. Its wall is lined on the inside by the ciliated former larval covering, which looks like a C-shaped mass, a ringed hole, within the larva. This C-shaped appearance can be attributed to the fact that the ciliated epithelium is darkly pigmented and opaque, except in the region of the piriform organ. What looks like an opening in the ring is undoubtedly the unpigmented piriform region, for the flagella always retreat inward from this side of the larva. After 10 minutes this ring gradually widens, the central clear area grows larger in diameter and the pigment seems to diffuse. This change probably results from the disintegration of the ciliated larval covering and of all the organs that served a useful purpose in the free-swimming organism.

Shortly after the apical epithelium emerges from the pallial furrow to form a fold which advances downward towards the adhesive plate, the apical organ loses its vesicular appearance and the tiny opening in its center begins to grow wider. During this time the apical epithelium can be seen advancing outward very slowly, as if it were being pulled and stretched by the new larval covering. This partially clarifies the observation that the upper margin of the old ciliated covering retreats inward very gradually, as the amount of material of the new covering is augmented very slowly by additions from the apical region.

Apparently the whole apical organ invaginates into the center of the C-shaped depression and undergoes histolysis, just as Prouho (1890) described for *Flustrella hispida*. He stated that the rudiment of the polypide, although it makes its appearance in this region later on, does not develop from the invaginated apical organ but rather from a meso-ectodermal disc formed independently from apical epithelium and from an internal mesodermal rudiment. The apical organ, like all the other organs that functioned in the free-swimming larva, degenerates after invagination.

Within an hour after fixation the adhesive plate, in the region where the vibratile flagella retreated, becomes deeply constricted by a notch, which can be seen for 4 or 5 hours after attachment (Fig. 11). One or two dark protrusions sometimes appear on the side opposite the notch. These are apparently the eye-spots pressing against the outer covering of the metamorphosing organism.

The larva passes into a stage known as the cystide about an hour after fixation. During this time the ectodermal walls secrete a thick covering, the ectocyst, which makes observation difficult. The further development is essentially similar to that described by Prouho (1890) for *Flustrella hispida*.

Eight or nine hours after fixation the rudiments of the polypide can be distinguished near the top of the elongating mass opposite the basal end and in the center of the now poorly-delimited C-shaped ring (Fig. 11). Twenty-four hours after fixation the zoöecium and the polypide within it are clearly recognizable. The

zoöcium itself is club-shaped, broader at the apical end where the lophophore will appear, and gradually narrowing towards the basal end, but flaring-out again at the point of attachment. At the end of 24 hours the young zoöids measure 0.6–1.0 mm. By 48 hours they have reached a length of 0.74–1.2 mm. and at 49 hours, 0.8–1.3 mm. The lower four-fifths of the body of the 24-hour zoöid is composed of transparent material derived from the adhesive plate; within it the tube-like polypide can be seen extending to the base of the holdfast. The apical end is made up of pigmented material, which forms a somewhat elongated, spherical mass that looks considerably like the unmetamorphosed larva. The transparent zoöecial wall encloses this spherical mass. At the extreme tip of the apical end two black spots mark the lophophores of the first and second individuals of the colony. The tentacles of the first are usually everted at the end of 28 hours; by this time a bud for the second individual is already well formed. Its tentacles are not everted, however, until approximately 48 hours have elapsed.

Since the lophophore and mouth of the first individual of the colony are formed in the region formerly occupied by the apical organ of the larva, there is a reversal of polarity through 180°. The oral end of the larva becomes the aboral end of the ancestrula of the colony.

The eversion of the internal sac and the events that take place during the "umbrella stage" occur so rapidly that the writer had to observe 40 or 50 larvae in order to get a clear grasp of the details. The "umbrella stage" can be seen best, when larvae attach to the lateral walls of a well-slide. On the other hand, the fimbriated nature of the holdfast can be seen only when larvae attach to the surface film. In this case the piriform organ is upward and the eye-spots are downward at the moment of fixation. Then the internal sac is suddenly everted upwards above the larva like a relatively huge balloon. The larva then turns, so as to bring the apical organ downward beneath the surface and the oral depression upward.

Larvae that attach to the surface film show no irregularities of development. The tentacles of the first individual and sometimes the second are everted in a normal manner, and they snap vigorously during active feeding. They are quickly withdrawn into the tentacular sheath, if the water is disturbed by jarring the table. When they are touched with a probe, their holdfasts cling tenaciously to it. Although the longevity of these larvae was not determined, on several occasions they were still alive 3 or 4 days after fixation. By this time the young zoöids had turned over, so that the tentacles protruded upwards. Strangely enough, they formed a continuous chain by means of thread-like projections that united each of them to the adjacent zoöids.

Although Visscher (1927) found that more bryozoan larvae affixed themselves to red test panels than to green, black or yellow ones and that none attached to white panels, the writer found no evidence that the larvae of *B. neritina* showed any preferences for pieces of red brick placed within the finger-bowls. They did prefer, however, the rougher surfaces of submerged test panels to the smoother parts, just as Visscher found. It appears likely that these larvae prefer to attach to organic material. In nature they frequently affix themselves to algae and to established bryozoan colonies. Although the presence of bacterial slime favors the attachment of most macroscopic organisms (ZoBell and Allen, 1935) and seems to be a prerequisite for the fixation of barnacles (Hillen, 1923), it does not have

any influence on bryozoan larvae (Scheer, 1945; Miller, 1946). Scheer (1945) found that bryozoan larvae would not attach to glass plates unless they had been submerged for at least two weeks; but they were influenced only by the abundant diatom population that had accumulated. The writer observed, during a three-week period in which larvae were shed daily in a finger-bowl, that 20 or 30 had attached to the body of a young *Styela* that was 2 or 3 cm. below the surface of the water, but none attached to the side-walls at this level.

### EXPERIMENTAL SECTION

#### *The effects of temperature on the responses of the larvae to light and gravity*

Grave (1930) found that the larvae of *Bugula flabellata* are photopositive during the greater part of their free-swimming period but become negatively phototactic shortly before fixation.<sup>1</sup> Mast (1911), too, found that other organisms show a similar reversal of phototropism from positive to negative as they approach the end of the pelagic stage. The larvae of *B. neritina*, however, under ordinary laboratory conditions of temperature and salinity were never observed to become negatively phototactic at any time; swarms of metamorphosed larvae could always be found on the lighted side of the container within an hour after they had been shed. Furthermore, they maintained a definitely negative geotaxis throughout the entire larval period and attached in large numbers just beneath the surface film. In some instances they attached to the sides of the container, but fixation always took place at or near the surface. Here, then, was a paradox! What factors in nature, not operative under the unusual conditions of the laboratory, could be responsible for causing larvae to attach near the bottom of the channel and in regions not exposed to the direct rays of the sun? At first, temperature seemed to be the most likely factor, for setting of the larvae occurs most abundantly during April and May (McDougall, 1943), when the sea water is relatively cooler than it is during the summer months. Again, it seemed likely that the more normal behavior of the Woods Hole species in the laboratory might be due not so much to specific differences as to lowered temperature, for the average temperature prevailing there during May, June, July, and August is 6° below that at Beaufort.

The hypothesis that temperature might be a controlling factor in bringing about the observed distribution was first tested during the summer of 1944. Although a reduction of temperature to coincide with that of the channel during April and May did not change the geotropism of the larvae, further cooling had a marked effect. The results of two experiments, selected from many similar ones with both stender dishes and vials, are summarized in Table I, which shows the responses of the larvae to both a descending and an ascending gradient. Counts were made at approximately 2.5–3.0-minute intervals during a 30-minute period, just long enough to reverse the geotropism of all the larvae. The vials were immersed in ice-water, and temperatures were taken at the bottom, where the water was 4 or 5° cooler than on top. Although no definite temperature could be determined as the critical one at which all the larvae were either geopositive or geonegative, nevertheless the data in this table and in others not recorded show that the greatest shift

<sup>1</sup> The terms, phototropism and phototaxis, will be used synonymously in this paper, without regard for their original meaning as described by Mast (1911, p. 253). These organisms may be either phototactic or photopathic, according to the older terminology.

from one response to the other occurred between 20 and 23.5° C. It can be noted that larvae exhibit a kind of inertia to a change of position, when either ascending or descending temperature gradients are employed. A change in geotropism is a function of time as well as of temperature. Thus, a drop of 13° had effected a change in only 37 per cent of the larvae; the remaining 63 per cent were affected by a change of only 3°. During the last 15 minutes of the experiment when the temperature remained constant (at 7°), 35 per cent of the larvae descended to the bottom. Similarly 65 per cent had not responded to a rise of temperature from

TABLE I

Temperature	Number of geopositive larvae	Percentage geopositive
23.0	0	0
14.1	16	34
10.0	17	37
9.5	19	41
9.0	22	48
8.5	24	52
7.5	25	54
7.0	30	65
7.0	33	71
7.0	34	74
7.0	36	79
7.0	39	85
7.0	46	100

Temperature	Number of geopositive larvae	Percentage geopositive
7.0	46	100
10.0	43	93
12.0	40	87
14.0	40	87
16.0	34	73
18.0	30	65
20.0	21	45
20.5	15	33
21.5	12	26
22.0	3 (active)	6.5

7 to 18°. During a subsequent 15-minute period, however, all but 6.5 per cent had reversed their positions in the vial, even though the temperature rose only 4°. Nine larvae, having attached at the bottom, could not respond.

Not only does a reduction of temperature change the geotropism of the larvae, but it also prolongs the free-swimming period. When normal sea water was cooled to 7 or 8° C., more than 80 per cent of the larvae were found to be active at the end of an hour, in contrast to only 6.7 per cent in the controls. In general, it may be stated that the average free-swimming period can be prolonged 2-3 hours by cooling the medium. Although the extreme limit to which the natatory period could be extended by treatment with cold water was not determined, nevertheless, in several instances 20-30 per cent were found to be active at the end of 5.5 hours. If we take into consideration the fact that the majority of larvae metamorphose at room temperature within 30-60 minutes after release from the ovicells, the effects of tem-

perature on the length of the swimming period are very marked. Marcus (1926c) made similar observations on the larvae of fresh-water bryozoans.

The motility of larvae can also be modified by temperature. When quiescent larvae, which had not yet metamorphosed, were taken from containers at room temperature (28° C.) and placed in water at 7–18°, they immediately became active. Although it is difficult to judge the degrees of motility merely by observation, apparently the greatest amount of activity was exhibited when the temperature was reduced to 16°. At 12° it was still considerable, but less than at 16°. At 7° motility was still more reduced and activity was largely confined to creeping movements.

Some organisms reverse their reactions to light when the temperature is reduced. Thus, *Euglena* (Mast, 1911), *Chromulina* (Massart, 1888), *Acartia* (Esterly, 1919) and haematococcus swarm spores (Strasburger, 1878) are photopositive at room temperature (18–20° C.) but become negative when the temperature is reduced (to 4–8° C.) Others, such as *Polygordius* (Loeb, 1905), change from positive to

TABLE II

Temperature	Percentage nearer the source of illumination	Percentage intermediate	Percentage farther from the source of illumination
22	35.0	31.2	33.8
20	73.0	4.0	23.0
19	19.4	16.0	65.0
19	31.0	14.0	55.0
19	81.0	0	19.0
17	76.2	14.3	9.5
13	80.0	20.0	0
9	80.0	0	20.0
7.5	0	51.7	48.3

negative with a reduction in temperature. *Daphnia*, on the other hand, shows no reversal of phototropism when the temperature is changed (Yerkes, 1900). In order to determine the effects of various temperatures on the phototropic reactions of the larvae of *B. neritina*, the following observations were made (Table II). Contrary to expectations based on casual observations, these experiments led to the conclusion that in cold water the larvae merely lose their otherwise intense positive reaction to diffuse daylight and become more or less indifferent, swimming back and forth towards and away from a source of illumination. Although B. H. Grave (1930) had observed that this activity precedes a definitely negative reaction in *B. flabellata*, the writer found that setting of the larvae of *B. neritina* occurred more or less at random in water at reduced temperatures.

Since larvae metamorphosed, apparently without any exceptions, on the lighted side of finger-bowls containing water at room temperature, for a time it was considered that they were photopositive at the time of attachment. When small vials were used, however, fixation occurred in 70 per cent of the cases either at random or at the center of the vials in circular masses just under the surface film. Approximately 50 per cent were on the half of the disc farther from the source of illumination. These settings seem to indicate that even at room temperature larvae become indifferent to light shortly before fixation. Since phototropism is correlated



with the amount of activity of the larvae, active swimmers are almost universally photopositive; as activity decreases, they lose their intense positive reaction to light and become more or less indifferent. Because a reduction of activity occurs only a very short time before metamorphosis, larvae under laboratory conditions usually cannot move very far from the lighted side of the container before fixation takes place. Even when larvae are made to react negatively to light by experimental prolongation of the free-swimming period, as described later, this reaction is never so intense as the positive one. Perhaps this observation explains why McDougall (1943) found the highest incidence of settings not in the most dimly illuminated part of the light box, but in the adjacent chamber where slightly more light was admitted.

### *Phototropism vs. geotropism*

Marcus (1926a) stated that the negative phototropism exhibited by the larvae of the phylactolaematous bryozoans shortly before fixation dominates a coexistent negative geotaxis and forces them to seek places of attachment at some distance beneath the surface of the water.

Since in nature both light and gravity act in approximately the same vertical plane, it might seem logical to identify a positive geotaxis with a negative reaction to light. The behavior of the larvae of *B. neritina* at reduced temperatures, however, demonstrates that they may become geopositive without showing a simultaneous negative reaction to light. The following experiments were set up to determine which of the two tropisms is dominant in this species.

TABLE III

No. of trials.....	12
Average temperature (mean).....	28.1 $\sigma = \pm 1.1$ (25-29 degrees)
No. of larvae.....	332
Percentage geopositive.....	28
Percentage intermediate.....	14.2
Percentage geonegative.....	57.8
Average time of exposure in minutes.....	8.9 $\sigma = \pm 1.8$ (8 to 10 min.)

If the response of the larvae to light were dominant to their geotaxis under given conditions, they ought to swim against a gravity gradient towards or away from a source of illumination, depending on their phototropism at the time. Consequently, on several different days larvae were made geopositive by treatment with low temperatures. Vials 16 cm. long and 1.8 cm. in diameter were used; some of them were cooled to 5° C., some to 6°, and others to 8°. They were then placed inside the light box, so that rays from a 100-watt bulb could enter only from the top. The source of light was 22 cm. from the bottom of the vials, where the larvae were swimming. As might be expected from the previous discussion of the indifference towards light exhibited by larvae at reduced temperatures, a positive phototropism under these conditions could not be demonstrated. Only 2.1 per cent of the larvae in 4 of the vials swam to the top; in the other three, none changed their positions as the result of the illumination. Six trials were then made with the light-box arranged so that rays could enter only from below. The light source was somewhat nearer the larvae in these cases, being only 6 cm. from the bottom of the vials. In all cases only negative results were obtained. The larvae did not swim away

from the bottom in a negative response to light. These experiments indicate, then, that larvae at low temperatures are either indifferent to light or that their positive geotaxis under these conditions is strong enough to counteract any reaction to light.

Different results were obtained with larvae kept at room temperature. When pipetted to vials, they were geonegative at the beginning of the experiment, and the vials were then placed in the light-box so that they were illuminated only from below. Many of the larvae swam downward. Counting was done immediately after the vials were removed and the results of 12 trials are recorded in Table III. Larvae were always observed to swim upwards, when the vials were removed from the source of light, in all the trials except one; they were never seen swimming in the opposite direction. Of the 332 larvae tested only 57.8 per cent remained geonegative after treatment. In the control vials, however, 91.2 were negatively geotactic. If due allowance is made for the probability that some of the larvae in the experimental vials had metamorphosed during treatment and could not respond, the contrast in percentages would indicate that at room temperature a positive phototropism is dominant to a negative geotaxis in approximately half the larvae. On the other hand, these experiments show that larvae respond positively to light with much greater readiness when they can swim along a horizontal plane than when they must swim downward against what appears to be a buoyancy gradient. In one of the vials illuminated from below all the larvae became geopositive and metamorphosed on the bottom, but, since this vial was exposed to alternate periods of intense and diffuse light, it seems likely that a positive geotaxis resulted from the prolonged activity induced by alternate periods of light. Subsequent experiments demonstrated that any condition that prolongs the activity of the larvae eventually induces a positive geotaxis.

#### *The effects of light intensity and darkness*

**Changing the intensity.** A rheostat was used to govern the intensity of a 100-watt bulb placed 8 cm. above a vial enclosed by the light-box. A ten-minute exposure to dim light was followed by a ten-minute exposure to intense light. Although 15 trials were made, all attempts to bring about a downward migration of the larvae by suddenly either increasing or decreasing the intensity of illumination gave no results. Apparently the larvae of *B. neritina* are not affected by changes of light intensity, as so many plankton organisms are.

**Intense light.** Since strong illumination often causes a reaction in many animals which is opposite to their behavior in diffuse light, experiments were performed by using both artificial illumination and direct sunlight. The artificial illumination was at least three times as intense as that employed in the experiments described above or those summarized in Table III. Sixteen-cm. vials of normal sea water were placed 3 cm. below and at the side of a 100-watt bulb, and a water jacket was used to maintain a constant temperature of 29° C. At the beginning of the experiment larvae removed to these vials were very active, intensely photopositive and were swimming on top. By the end of 30 minutes, however, 41.8 per cent of the larvae in the experimental vials had descended to the bottom, whereas only 9.8 per cent became geopositive in the controls. Apparently a fairly high percentage of larvae at room temperature is negatively phototropic to intense light. The data obtained with strong sunlight, although comparable, were not clear-cut enough to

warrant publication, since temperature may have modified the results somewhat. Nevertheless, there is some evidence that strong sunlight causes larvae to move downward from the surface to regions of lower light intensity

Both strong sunlight and artificial illumination induce larvae to move along a horizontal plane away from the side where the rays enter the medium obliquely. Although strongly photopositive at first, by the end of 20 minutes all the larvae (in three vials) had moved away from the bulb to the distal side. Those exposed to sunlight remained photopositive much longer; but by 1.5 hours all had become photonegative in two of the vials and only 5 per cent remained photopositive in the third. In the latter, 45 per cent were intermediate and 50 per cent showed a negative reaction. Here again there is evidence that light is more effective in causing larvae to move along a horizontal plane than up or down, either towards or away from a source of illumination.

The negative reaction of larvae to intense light would seem to be significant. Even a casual observation of the distribution of various species of *Bugula* near the water line on piers and docks will show a preponderance of colonies on the north side of objects and in shaded places. The evident fact that in nature the majority of larvae are negatively phototropic at the time of attachment may be explained on the basis that they swim away from regions receiving the direct rays of the sun after a certain amount of exposure to intense light, a behavior that they do not show in a laboratory illuminated only by diffuse light. The opinion of Esterly (1919) that the physiological state of animals is changed, when they are removed from the ocean to the laboratory, would seem to be true only if all the conditions prevailing in their former surroundings are not exactly duplicated. Since the light-box used by McDougall (1943) was placed only 6 inches below low water mark, the amount of absorption of the sun's rays was probably insignificant; hence it should be expected that the larvae would be photonegative as described. Whether larvae far beneath the surface at the time of attachment show a negative phototaxis like those near the water-line can only be conjectured. Since absorption would considerably reduce the intensity of sunlight received by the lower regions, it might be expected that they would be either photopositive or indifferent, like those in the laboratory under conditions of diffuse daylight.

**Darkness.** Darkness sometimes inhibits the movements of animals and may change their reactions to both light and gravity. The work of Holmes (1905) on *Ranatra* led him to conclude, "The causes that produce the negative reaction [to light] are, as a rule, those which lead to diminished activity and excitement. Cold, exposure to darkness, and the quieting effect of contact stimuli lead to a condition of lessened excitability and, perhaps as a result of this, to a negative reaction to light" (p. 317). Mast (1911) obtained a reversal of phototropism in the larvae of *Arenicola* by subjecting them to a variety of agents, such as diluted and concentrated sea water, magnesium chloride, atropin, sodium hydrate and ammonia. He believed that depressants merely hasten the onset of that distinct physiological state, usually accompanied by a negative reaction to light, which larvae normally attain as they grow older. Esterly (1919) found surface specimens of *Acartia* to be negatively geotropic in diffuse light but positive in darkness at the same temperature, although deep sea specimens (at room temperature) showed no change in geotaxis. Marcus (1926a) found that the larvae of *Plumatella fungosa*

are negatively geotactic in nature, both in darkness and in light, throughout the entire natatory period.

In a series of experiments, larvae of *B. neritina* were placed in stender dishes at room temperature and removed to a darkroom for a period of half an hour. When the containers were again returned to diffuse daylight, the larvae showed no change in their reaction to light. Neither was there any change in geotaxis. Similarly, larvae kept at reduced temperatures showed no change of tropisms, when they were returned to diffuse daylight after a 30-minute period in the darkroom.

On the other hand, there is some evidence that darkness reduces the activity of the larvae and decreases the length of the free-swimming period. Three experiments were performed by subjecting larvae to conditions that, in diffuse daylight, ordinarily prolong the natatory period well beyond 2 hours. Two vials, containing sea water diluted to a density of 1.010 grams/cc., were placed in the darkroom. When the first vial was inspected at the end of an hour, all the larvae were found to be active; but at the end of 2 hours only 28 per cent were active, whereas 55.8 per cent were still swimming in the controls. (For the effects of diluted sea water see Table V.) In the second vial none were active at the end of 2.5 hours, although 27.5 per cent of those in the controls were still motile at the end of 4 hours. The third vial contained normal sea water which was kept at a temperature reduced sufficiently to cause prolonged activity in diffuse daylight. None of the larvae in this vial were active at the end of an hour. Although more experiments are needed, the results indicate that darkness probably reduces the length of the natant period in this species.

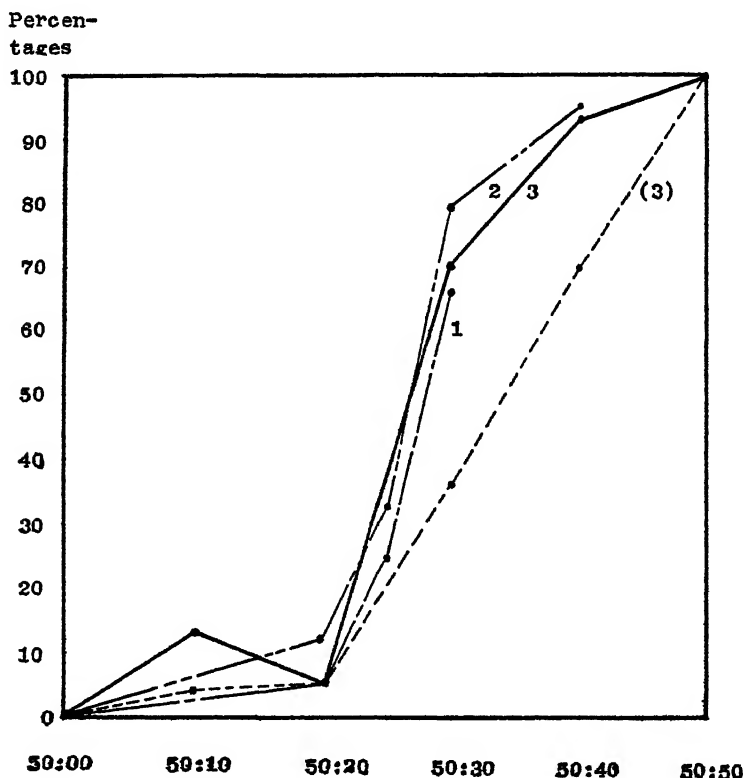
*The effects of salinity on the duration of the free-swimming period and the responses of the larvae to light and gravity*

Since *Bugula* grew profusely near the laboratory at the mouth of the Newport River, it was surprising that adult colonies could not be detected near the water line on pilings and docks only three-quarters of a mile up-stream. The few that were found were growing near the bottom of objects that could be raised to the surface. A sample of the water in this region, taken about an hour after the tide had turned and sea water had begun running up-stream, was found to have a specific gravity of 0.0116 (28° C.), whereas the lowest of 25 tests made on water near the laboratory gave a reading of 1.0164.<sup>2</sup> It seemed likely that this difference in density, although small, would not represent the maximum, since at low tide the amount of fresh water would undoubtedly be greater and the density would be correspondingly lower.

That the salt content of the water might affect the geotropic responses of the larvae and determine their ultimate place of attachment seemed to be the only explanation for the observed distribution. Furthermore, on several occasions during the summer of 1944 the writer had noticed that larvae seemed to metamorphose in an abnormally short time when concentrated sea water was added to a well-slide. Consequently, a series of experiments were performed by using various concentrations of sea water below and above the normal range. Since 16 out of 25 samples of sea water in the region of the laboratory showed a specific gravity of 1.0200–

<sup>2</sup> In order to avoid unwieldy decimals in Table V, temperature corrections were not made. The maximum deviations due to temperature fluctuations would not exceed 0.00076 gr./cc.

1.0226, this may be considered approximately the normal range during the time the experiments were performed. These samples were taken on different days at both high and low tide during a three-week period. The highest reading was 1.0226 and the lowest was 1.0154; seven readings showed a prevailing density of 1.016–1.0173 gr./cc. during a period of especially heavy rainfall in the third week of August. Gutsell (1930), however, found a higher average density during the



GRAPH I. Dilutions of sea water (50 cc.) with distilled water

Lines 1 and 3 (solid) show the percentages of geopositive larvae at the end of 1.5 hrs. Line 2 (solid) shows the number geopositive at 5.5 hrs. Dotted line (3) shows the number active at the end of 1.5 hrs. in vials used for solid line 3.

years his readings were taken (1924–28), even if temperature corrections are made for those listed above; at that time the salinity in this region approximated 30 parts per mille, but occasionally ran as high as 38 or as low as 6 parts per mille (20° C.).

Graph I shows the results of 11 experiments carried out by diluting 50 cc. of sea water with 10 to 50 parts of distilled water. The temperatures were read at the time the densities were taken, and the observations were carried out on the same day between 11 A.M. and 4:30 P.M. (E.S.T.). The following table shows the densities in gr./cc. of the various dilutions used. (The writer found that the

density of a sample of sea water rose approximately 0.00017 gr./cc. for each degree (C.) drop in temperature.) It will be noted that dilutions of 50:10 and 50:20 had but little effect on the number of larvae that became geopositive. The number of geopositive larvae began to increase at dilutions greater than 50:20 and grew progressively larger as the salinity decreased. A decrease in salinity likewise increased the average length of the natatory period. Since the water three-quarters of a mile upstream had a density of 1.0116 gr./cc., a dilution slightly greater than 50:30, its low salt content may explain the location of *Bugula* colonies near the bottom of the stream.

TABLE IV

Dilution	Temperature	Gr./cc.
50:50	29.0	1.0101
50:40	29.5	1.0108
50:30	29.9	1.0119
50:20	30.0	1.0149
50:10	30.0	1.0169
50:00	29.7	1.0228

Since random samples of the laboratory supply of sea water, taken from the mouth of the Newport River, showed fluctuations caused not only by high and low tide but also by the amount of rainfall, more extensive data were obtained by using sea water diluted or concentrated to a definite specific gravity as determined by means of a very sensitive hydrometer. Care was taken to fill the vials to the same level (about 5.5 cm.) and to add approximately the same amount of water, when larvae were pipetted from the finger-bowls. The addition of such small amounts of water to the vials did not seriously increase the densities in the lower ranges, where experimental results were most pronounced. The introduction of non-motile larvae, which cannot respond to treatment, was avoided by first transferring larvae from the finger-bowls to a stender dish of fresh sea water before introducing them into the vials. Since the active ones invariably swam to the lighted side of the dish within a minute or two, large numbers could be transferred without difficulty. Because quiescent larvae sometimes become active after a few minutes of observation, counts had to be made 3 or 4 times for each vial. The difficulty of determining the number of larvae that were active at a given time necessitated the use of only small populations for each vial; the ideal number was found to be about 20 to 30. For these experiments 2,358 larvae were counted during a 5-week period. The results are summarized in Table V.

Larvae kept in water having a specific gravity of 1.010 to 1.012 were extremely active for an abnormally long time, definitely photopositive during the time of greatest activity and geonegative during the early stages of the natatory period. The majority, however, descended to the bottom towards the end of the free-swimming period. These larvae were visibly smaller than those that had a shorter period of activity and measured only 0.14 mm. in diameter as contrasted with the average size, 0.20–0.22 × 0.27–0.30 mm. Since this decrease in volume was found to be correlated with a prolongation of the free-swimming period, it seems likely that the activity of the larvae resulted in a conversion of stored food material into

waste products of metabolism that passed out into the surrounding medium. It is not improbable that the lipid granules observed by Barrois (1877) and others constitute a reserve food supply for those forms of bryozoan larvae without an alimentary tract. Furthermore, these larvae had extruded an orange colored pigment that gave a characteristic color to the water in the bottom of the vials.

TABLE V

Specific gravity	Number of larvae	Percentage geonegative in hours				Percentage intermediate				Percentage geopositive				Percentage active					
		1/2	1	2	4	1/2	1	2	4	1/2	1	2	4	1/2	1	2	4	Number larvae	6
1.010	203	44	30	8	6	18	28	38	6	38	42	54	88	100	87	67	39	44	16
1.011	126	72	42	34	22	8	32	36	10	20	26	30	68	97	88	52	38	26	8
1.012	135	85	76	77	65	5	6	6	5	10	18	17	30	87	55	38	27	39	10
1.013	207	98	92	83	58	0	0	0	7	2	8	17	35	59	55	30	9	54	5
1.014	85	96	94	93	86	3	2	2	1	1	4	5	13	66	26	10	7	43	9
1.015	137	88	89	90	93	11	4	4	0	1	7	6	7	65	31	4	1	44	0
1.016	168	84	87	90	90	7	4	1	1	9	9	9	9	28	10	3	0	96	0
1.017	143	88	89	89	90	2	2	2	1	10	9	9	9	26	9	4	2	187	3
1.018	112	92	92	93	93	1	1	0	1	7	7	7	6	32	7	2	1	32	0
1.019	148	98	97	97	97	0	0	0	0	2	3	3	3	12	2	0	0	85	0
1.020	159	95	98	96	96	2	1	1	0	3	1	3	4	21	6	1	1	26	0
1.021	171	97	90	94	93	1	3	3	2	2	7	3	5	19	5	3	1	95	2
1.022	97	95	—	—	94	0	—	—	0	5	—	—	6	10	5	0	0	63	0
1.023	104	83	85	86	88	10	5	5	3	7	10	9	9	25	5	2	0	62	0
1.024	115	69	66	65	65	11	11	12	13	20	23	23	22	30	9	1	0	43	0
1.025	214	15	16	16	16	12	10	11	11	73	74	73	73	8	3	1	0	57	0
1.026	134	7	5	6	6	6	10	7	4	87	85	87	90	13	9	3	2	39	0
Total	2358																		

Temperature of sea water for above—25–29°C.

Temperature at which hydrometer readings were taken—27.5–29°C.

The larvae themselves were orange-brown, whereas those kept in water of higher specific gravities always appeared black. Lillie (1909) found a similar pigment in the larvae of *Arenicola*; this, he said, is derived from the egg cell and normally disappears during later larval stages. Prolonged muscular activity caused this pigment to leave the cells and color the surrounding medium with a faint yellow tinge.

Larvae placed in sea water at densities between 1.016 and 1.022 gr./cc. (the normal range) behaved quite differently from those in the lower densities. An average of 92.1 per cent remained geonegative at the end of 4 hours, and 90 per cent at the time of fixation; in vials containing sea water at a density of 1.010 gr./cc., however, only 6 per cent were geonegative at the end of 4 hours and at the end of the free-swimming period. Furthermore, those kept in sea water at densities between 1.016 and 1.022 gr./cc. had considerably abbreviated natatory periods when contrasted with larvae in water at lower densities. At the end of 4 hours the average percentage of active larvae in 44 vials containing water at the higher densities (1.016–1.022) was 0.71 per cent whereas 34 per cent were active at densities from 1.010 to 1.012 gr./cc. inclusive.

In sea water more concentrated than 1.022 gr./cc. the percentage of larvae that descended to the bottom increased with the density. In these cases, however, a geopositive reaction was not preceded by a prolonged period of activity. At densities of 1.025 and 1.026 gr./cc. only 10.5 per cent were active at the end of half an hour, whereas 22.5 per cent were active in concentrations between 1.016 and 1.024 gr./cc. and 100 per cent were active at a density of 1.010 gr. cc. At a density of 1.026 gr./cc. only a few larvae were active after 10 minutes; they sank passively to the bottom almost as soon as they were introduced into the vials, and generally only feeble movements could be detected afterwards. Evidently the factors that caused these larvae to descend to the bottom were quite different from those that brought about a similar positive geotaxis in sea water of low salt concentration. In the latter, the larvae swam slowly to the bottom after they had been active a very long time. When the effects of concentrated sea water were observed under the microscope, it was found that larvae metamorphosed in a remarkably short time in almost epidemic proportions. It seems unlikely that the organisms were effected by any change in the pH produced by diluting or concentrating the medium, for subsequent tests showed that a dilution to 1.010 gr./cc. caused the pH to drop only 0.08 and a concentration to 1.026 increased the pH only  $0.52 \pm 0.1$ .

#### *The development of larvae at various salinities*

Vials containing larvae that had already formed zooids were inspected at the end of 24 hours. Those in more concentrated sea water were slightly larger than normal. All of them had their tentacles everted and snapping actively at this time. These zooids were attached securely to the substrate.

Those in vials containing sea water at specific gravities of 1.016 and 1.017 were normal in every respect at 29 hours, except that they did not withdraw their tentacles as a reaction to mechanical jarring, as they normally do in higher salt concentrations. Those that developed at a density of 1.015 gr./cc. had their tentacles everted, but these were protruding at an angle of  $90^\circ$  to the normal position. Apparently these organisms were entirely incapable of withdrawing them, for mechanical jarring had no effect during the 30 minutes they were under observation.

In vials containing sea water at densities below 1.015 gr./cc. development was poor. There was a marked reduction in size, and none had their tentacles everted (29 hours). Nevertheless, even those that metamorphosed in water having a density of 1.010 gr./cc. had normally formed internal organs that moved spasmodically at times, and most of them had a bud for the formation of a second zooid. Again it seems likely that the poor development of these zooids can be attributed to an insufficiency of stored food at the time of metamorphosis, caused by their prolonged activity. Apparently some of the larvae in water at a density of 1.010 had failed to metamorphose; and many of them did not adhere to the bottoms of the vials, for they could be easily dislodged by a stream of water.

#### *Salinity vs. osmotic pressure*

In order to determine whether the observed effects were due to the salt content or to the osmotic pressure, sea water was diluted to a density of 1.010 gr./cc. and sucrose was added until the specific gravity reached 1.026. Since larvae in sea water at a density of 1.010 gr./cc. remained active for an unusually long time, it was considered logical to conclude that osmotic pressure would not be a factor, if the



larvae showed the same behavior after the pressure had been raised by sucrose. On the other hand, if an abbreviation of the natatory period took place, it could be concluded that this effect was caused by an increase in osmotic pressure.

Since subsequent tests showed that similar sucrose solutions had a freezing point depression equal to that of sea water at a density of 1.014 (see Table VI), vials con-

TABLE VI

Density in gr./cc. of original sea water	Substance added	Final density gr./cc.	Freezing point depression	pH
1.010	NaCl	1.017	1.74	7.6 $\pm$ .10
1.010	CaCl <sub>2</sub>	1.017	1.36 $\pm$ .02	7.07 $\pm$ .07
1.010	KCl	1.017	1.67 $\pm$ .11	7.61 $\pm$ .05
1.010	MgC <sub>2</sub>	1.017		7.58 $\pm$ .14
1.020	CaCl <sub>2</sub>	1.026	2.20	7.85
1.010	Sucrose	1.026	1.25 $\pm$ .02	7.2 $\pm$ .20
1.010	Sucrose	1.017	—	6.7
1.010	Glucose	1.026	1.41	6.2
Distilled	NaCl	1.026	—	6.3
1.017	None	1.017	1.51 $\pm$ .07	7.67 $\pm$ .02
1.024	None	1.024	2.06 $\pm$ .03	8.25
1.026	None	1.026	2.21 $\pm$ .07	8.25 $\pm$ .05
1.010	None	1.010	.96	7.60

$\pm$  indicate repeated experiments.

*Osmotic pressures of sea water at various concentrations.* Temperature = 27.5–29.0° C.

Specific gravity	Freezing point depression	Pressure in atmospheres
1.010	0.96	11.56
1.011	1.09	13.12
1.012	1.11	13.36
1.013	1.17	14.08
1.014	1.26	15.16
1.015	1.36 $\pm$ .03	15.36 $\pm$ .36
1.016	1.46 $\pm$ .07	17.56 $\pm$ .84
1.017	1.51 $\pm$ .07	18.16 $\pm$ .84
1.018	1.62 $\pm$ .01	19.48 $\pm$ .12
1.019	1.62 $\pm$ .01	19.48 $\pm$ .12
1.020	1.67 $\pm$ .05	20.08 $\pm$ .60
1.021	1.87	22.48
1.022	1.87	22.48
1.023	1.95	23.44
1.024	2.06 $\pm$ .03	24.75 $\pm$ .35
1.025	2.10 $\pm$ .02	25.23 $\pm$ .25
1.026	2.21 $\pm$ .07	26.55 $\pm$ .96

$\pm$  indicate repeated experiments.

taining water at this density may be considered controls. In the sucrose solutions 45.3 per cent of the larvae were active at the end of 4 hours and 94 per cent had become geopositive; in the controls only 6.9 per cent were active and 13 per cent had descended to the bottom. It seems apparent that an increase in osmotic pressure by a non-electrolyte does not have the same effect as the addition of ions normally pres-

ent in sea water. These results are in agreement with the observations of Loeb (1900), for he found that marine organisms are easily affected by a disturbance in ionic balance, but are practically independent of osmotic pressure.

Larvae placed in a similar solution of glucose and sea water behaved quite differently, for they immediately sank to the bottom and remained immobile. When the pH values were obtained for similar solutions made with the same glucose, it was found that they were somewhat acid when freshly made (5.8-6.2); by the end of 24 hours the pH had fallen to 4.5, and by 48 hours had reached 3.4. Since the solutions used at Beaufort were made the day before the experiments were performed, presumably the anomalous behavior of the larvae can be attributed to an originally high degree of acidity, which was further enhanced by bacterial action. The sucrose solutions, however, showed no great acidity, for they dropped to only 6.5 at the end of 24 hours. The effect of pH on the behavior of other species of larvae investigated recently at Woods Hole will be described in another paper.

*The effects of various ions on the length of the free-swimming period and the metamorphosis of the larvae*

Since the experiments just described gave presumptive evidence that variations in salt content played a far more important role than an accompanying increase or decrease in osmotic pressure, the following observations were made to determine the specific effects of the four ions most abundant in sea water by using the chlorides of the metals. For comparing the effects of normal sea water with sea water having a high concentration of only one of the ions, a "fundamental solution" was made by diluting sea water to a specific gravity of 1.010. Since this concentration contains ions in the same proportion as normal sea water and is capable of supporting prolonged larval life, it may be presumed that any modification of the behavior of larvae can be attributed to the ions that were added. The osmotic pressures and pH values of similar solutions are given in Table VI.<sup>8</sup>

**Effects of sodium chloride.** Larvae placed in pure solutions, made by adding sodium chloride to distilled water until a specific gravity of 1.026 was obtained, sank to the bottom immediately, became immobile and turned completely white within three minutes, so that they could be seen easily only against a black background. When viewed under the microscope, the larvae were observed to undergo metamorphosis very rapidly. Their white appearance was due largely to the enormous amount of milky white holdfast material ejected and to an inward migration of pigment granules from the surface of the larvae. After the initial stage of metamorphosis had begun, the succeeding stages occurred somewhat more slowly than in normal sea water. Most of the larvae were shrunken and appeared to be about two-thirds normal volume. At the end of 3 hours they looked like irregular pieces of white jelly; their edges were ragged and protoplasmic threads protruded from their surfaces. Those on the bottom of the vials were not anchored securely and could be easily dislodged by directing water from a pipette against them; and, after being dislodged, they floated to the top. Evidently metamorphosis had decreased their density. Similar results were obtained with solutions made by adding enough sodium chloride to distilled water to raise the specific gravity to only 1.010, but in this case

<sup>8</sup> Densities were measured in connection with ion effects because equipment was easily available for such measurements and these effects were to be compared with other results in which density measurements had been used.

the larvae did not take on a characteristic white appearance until 30 minutes had elapsed. Metamorphosis, therefore, did not occur so rapidly.

Finally a "fundamental solution" was made and raised to a specific gravity of 1.017 by sodium chloride. When first admitted to the vials, the larvae sank to the bottom immediately; but after a few minutes almost all had ascended to the top and were swimming actively. At the end of an hour 16 per cent were still active and 72 per cent had descended to the bottom and remained there, whereas in normal sea water of the same osmotic pressure only 5 per cent were active and 3 per cent were geopositive. The first reaction of larvae placed in a new environment seems to be what Marcus (1926a) called a "schreckreaktion"; they sink down passively and remain motionless for a few minutes. This reaction, when observed under the microscope, was found to be preceded by a violent constriction of the organisms in the region of the equator. Similar behavior was reported for the nauplii of *Balanus amphitrite* by Edmondson and Ingram (1939).

**Effects of potassium chloride.** The first solution was made hypertonic to the medium from which the larvae were taken by adding potassium chloride to normal sea water (density, 1.020 gr./cc.) until a specific gravity of 1.026 was obtained. The second was made isosmotic to sea water having a density of 1.020 by adding the chloride to a "fundamental solution." In both solutions the larvae sank immediately to the bottom and became milky white as they left a trail of black pigment granules behind them. After reaching the bottom, a few remained slightly active for about 10 minutes. They were somewhat more active in the second solution of lesser density, moving slowly and aimlessly on the bottom, although the cilia were beating in an almost normal manner. Perhaps the effective stroke was reduced enough to prevent active movement. After an hour their appearance was similar to those in the pure solutions of sodium chloride, except that metamorphosis did not occur. Potassium chloride had a similar effect in causing the loss of pigment from already metamorphosed larvae that were floating on the surface of finger-bowls, but in this case the pigment streamed out below the larvae and sank to the bottom; the larvae themselves remained on the surface. Lillie (1909) observed a similar loss of pigment in *Arenicola* larvae placed in potassium solutions (cf., also, Chambers and Reznikoff, 1926).

**Effects of calcium.** Larvae placed in distilled water raised to a specific gravity of 1.010 by calcium chloride went to the bottom and ceased movement abruptly. At the end of an hour, they looked orange-brown and flattened like oblate spheroids, caused by a shortening of the oral-apical axis; they closely resembled larvae that had a prolonged natatory period in sea water reduced to a density of 1.010 gr./cc., except that those in the calcium chloride solution were larger than normal, measuring  $0.29-0.30 \times 0.30-0.34$  mm. When other larvae were placed in a solution made hypertonic to the medium from which they were taken (sea water at a specific gravity of 1.020) by the addition of calcium chloride until a density of 1.026 gr./cc. was reached, they likewise sank to the bottom but remained slightly active for 2 hours. The cilia and vibratile flagella continued to beat rapidly, even though the larvae themselves did not move. Although the holdfasts had been ejected in many of them, normal attachment did not take place and the larvae continued to move aimlessly around with their holdfasts trailing behind them. They were badly fragmented and considerably reduced in size, measuring only 0.18-0.19 mm. along their

longer diameter. Finally, calcium was added to the "fundamental solution" to bring the specific gravity up to 1.017. Larvae in this solution showed a marked prolongation of the free-swimming period. In one vial 20 per cent were still active after 8 hours. In another, 2 larvae were still active after 12 hours, a strikingly long time in comparison with the normal period of 30-60 minutes. These larvae, in contrast to those in isosmotic sea water (specific gravity, 1.015), behaved quite differently from the latter. Not only did they have a decidedly longer natatory stage, but all, without exception, became geopositive at the end of the free-swimming period; only 8 per cent of those in isosmotic sea water became similarly geopositive. These larvae in the calcium chloride solutions behaved like those in normal sea water diluted to a density of 1.010 gr./cc. Like the latter, they remained geonegative, swimming just under the surface, during the greater part of their larval period, before they descended to the bottom. There was one significant difference, however, for they remained active much longer than those in diluted sea water. Furthermore, they did not attach rigidly like the latter.

**Magnesium chloride.** Sea water diluted to a density of 1.010 gr./cc. was raised to a specific gravity of 1.017 by magnesium chloride, so as to approximate the osmotic pressure of normal sea water. Larvae in this solution showed an initial shock reaction and sank to the bottom. They recovered rapidly, however, swam to the top and continued to swim feebly at the surface for half an hour. They showed a pronounced tendency to coalesce in groups. Lillie (1909), who observed a similar coalescence of the larvae of *Arenicola* in high concentrations of magnesium chloride, attributed this behavior to a loss of muscular contractility without an accompanying decrease in ciliary movement. By half an hour, active swimming movements had ceased. Whenever larvae at the surface became quiescent, jarring the vials caused them to become active again. Each time this was repeated, several larvae swam downward, after a brief period of swimming at the top, and remained on the bottom. By the end of 40 minutes, all the larvae had descended to the bottoms of the vials. Although they were not swimming, these larvae lying inert on the bottom had failed to attach and metamorphose. Five hours and 45 minutes after they were placed in these solutions, pipettes-full of larvae were removed and added to normal sea water; the majority began to swim actively again.

### *Mechanical agitation*

Although the vertical distribution observed by McDougall (1943) could not be attributed to modifications of any of the factors thus far investigated, since they had to be more extreme than any prevailing in nature, nevertheless one correlation became increasingly evident. Prolonged activity of the larvae was always associated with a positive geotaxis. This was true, whether induced by a reduction of temperature or salinity or by an excess of calcium over the other ions present in sea water. Furthermore, mechanical jarring, by activating the larvae in solutions of magnesium chloride, had caused them to descend to the bottom. Agitation by waves and wind seemed to be the stimulus provided by nature for bringing about a positive geotaxis. Moreover, experiments on other animals have shown a correlation between mechanical agitation and a change in physiological state. Rogick (1939), for instance, found that excessive handling of the larvae of fresh-water bryozoans abnormally prolonged and interfered with metamorphosis. Similarly, contact

stimuli frequently change the phototropism of organisms, as evidenced by the experiments of Holmes (1905) on *Ranatra*, Parker (1902) on *Labidocera* and Towle (1920) on *Cypridopsis*. The writer likewise observed that the larvae of *B. neritina* became negatively phototropic for about a minute after they were ejected from a pipette.

To determine the effect of mechanical agitation on larval behavior, an air jet from a pipette was directed against the surface of the water whenever larvae showed a tendency to become quiescent. This caused them to swim actively again. In brief, 19 per cent were active and 62.5 per cent were geopositive in the experimental vial at the end of 4 hours, whereas only 11 per cent were active and 11 per cent were geopositive in the control. In view of the correlation that exists between mechanical agitation and swimming movements on the one hand and between prolonged activity and a positive geotaxis on the other, it seems likely that further experiments would yield similar or even better results. Since the writer never observed that larvae were liberated in darkness under laboratory conditions, it is difficult to explain the observation that in nature they attach as readily at night as in the daytime (Edmondson and Ingram, 1939; Edmondson, 1944), unless it is assumed that these organisms, liberated by light, remain active through mechanical agitation during the day and part of the night. Certain experiments performed by the writer indicate that light, as well as mechanical agitation, may serve as a stimulus to movement.

#### DISCUSSION

##### *The nature of geotaxis in B. neritina*

Mechanical agitation is one of the factors existing in nature that is not generally duplicated under laboratory conditions, even though all the other factors belonging to the habitat of an organism seem to be present. The abnormally tranquil surfaces prevailing in the laboratory are not at all comparable to those encountered by larvae when they reach the surface of the ocean and are constantly buffeted about by waves and wind. Presumably larvae in their natural surroundings have a somewhat longer free-swimming period than those under laboratory conditions. The action of waves and wind prevents them from attaining that degree of quiescence which is a natural prerequisite for metamorphosis. Thus they remain active; and, as a result of prolonged activity, they descend to regions where a greater degree of calmness prevails. Crevices in rocks and pilings undoubtedly afford a certain amount of protection against the buffeting action of waves; hence a natural explanation may be given for the preponderance of young zooids that can be observed in grooves and in holes bored into test panels, after they have been submerged for a few days. At the present time there cannot be given a completely empirical explanation that will clearly demonstrate the *modus operandi* of the various factors that bring about a positive geotaxis under natural conditions.

Geotaxis in marine larvae cannot be assigned to the same category of responses as the other tropisms. Phototropism, for instance, whether it be positive or negative, is an active kind of response. On the other hand, what we call a positive or negative geotaxis can be resolved into other mechanisms. A negative geotaxis can be attributed to ciliary action, to buoyancy or to a more abundant supply of oxygen near the surface. It has sometimes been identified with a positive phototropism. Many aquatic larvae, such as those of *Arenicola* (Lillie, 1901) or of the phylacto-

laematous Bryozoa (Marcus, 1926c), are specifically denser than the surrounding medium. They can keep afloat only by ciliary movement, and when the cilia are injured they sink to the bottom. Others, apparently, are lighter than the surrounding medium and are buoyed up passively by Archimedes' Principle. Hora (1930) stated that some organisms can decrease their specific gravity by a reduction of the abdominal cavity; others, such as the larvae of *Megalophrys*, have hydrostatic organs. Thus their rising or sinking depends upon their density. Similarly, Visscher (1928) observed that the cyprid larvae of *Balanus eburneus* have a fatty substance in the anterior of their bodies that acts like a buoy in holding them near the surface; this lipoid material gradually disappears towards the end of the pelagic period. McDougall (1943) found that the setting of these larvae occurred in greatest abundance at a point some 5 or 6 feet below the surface. In another species, *Chthamalus fragilis*, however, he found that the lipoid droplets do not disappear towards the end of the free-swimming period; the highest incidence of settings of this species was very near the surface, and no attached larvae were found more than 6 inches below mean low water. Evidently buoyancy, or the lack of it, can determine the vertical distribution of certain organisms. In the cyprid larvae just described phototropism is not a factor in determining the ultimate place of fixation, for Visscher (1928) found that both species are photonegative just before attachment. A positive geotaxis should not be confused with a negative phototropism, as has been done sometimes in the case of bryozoan larvae. Sometimes, at least, the two tropisms are dependent upon entirely different mechanisms.

There are several possible explanations for differences in the behavior of the larvae of *B. neritina* in warm and cold water. It might be assumed that, since oxygen would be more abundant near the surface, the larvae would collect there when the temperature is high, because they are unable to obtain sufficient oxygen to support their increased metabolic activity, if they venture far from the surface film. The possibility that a negative geotaxis may be simply a positive response to oxygen in the case of free-swimming larvae has been suggested by Marcus (1926a) as a plausible explanation for the pelagic habits of certain marine Bryozoa. In his experiments on fresh-water Bryozoa (1926c), however, he could not obtain conclusive evidence that the larvae move in the direction of an increasing oxygen gradient. The writer, also, found no confirmation of such a theory. When a vial of sea water was inverted over a petri dish with the bottom supported on two slides so that oxygen could enter only from below, the larvae showed not the slightest tendency to move downward. Neither did they surround an air bubble that was on the side of the vial opposite the source of illumination. Furthermore, it would be difficult to explain a positive response to gravity, when the temperature is lowered, on the assumption that the negative geotaxis exhibited in warm water is merely a positive response to oxygen. Even though the oxygen content of water at reduced temperatures might be sufficient to maintain the lowered metabolic requirements of larvae at various depths beneath the surface, their movement in a direction of a slightly decreasing oxygen gradient would require explanation.

At first sight, it would seem logical to attribute the rising or sinking of the larvae to ciliary action alone, if it is assumed that these organisms are denser than the surrounding medium. Their descent in hypotonic solutions only after prolonged swimming might be attributable to ciliary fatigue. Since a reduction in

temperature decreases ciliary movement in the gills of *Mytilus* (Gray, 1923) and since hypertonic solutions (Gray, 1922), sea water having a pH below 5.5–6.0 (Gray, 1920) and solutions of sodium and potassium (Gray, 1920) have an even more deleterious effect, the sinking of the larvae of *B. neritina* under almost identical conditions might seem to be due solely to attenuation of ciliary action. The prolonged negative geotaxis of these organisms in solutions containing calcium concentrated to a certain optimum would seem to be correlated with the favorable effects of this ion on ciliary action, as described by Lillie (1901) for *Arenicola*. On the other hand, it might be expected that ciliary action would be maintained better in solutions containing an excess of potassium ions than in a similar sodium solution of approximately the same pH and osmotic pressure, for the latter has a more pronounced inhibitory effect on the cilia of *Mytilus* (Gray, 1920) or of veliger (Mayer, 1911) or *Arenicola* (Lillie, 1901) larvae.

The larvae of *B. neritina*, although they swim sluggishly in a horizontal plane just beneath the surface film in warm water, give no indication that they remain there only by active swimming. In fact, both dead and metamorphosed larvae were always found floating at the surface, and obviously ciliary action cannot account for this. They seem rather to be buoyed up by the medium. If this is true, they would differ from the phylactolaematous Bryozoa, which maintain themselves at the surface only by active swimming and sink downwards when injured by heat or by other unfavorable conditions (Marcus, 1926c). Again, larvae swam without exception towards the side of a vessel nearer the source of diffuse daylight, whereas only 42.2 per cent swam downward when they were illuminated only from below. This difference in behavior would seem to indicate that ciliary action is much more effective in moving them along a horizontal plane than vertically downwards against what appears to be a buoyancy gradient. Presumably the orientation of their long axes determines their direction of motion. It cannot be assumed, however, that larvae at room temperature do not swim beneath the surface because their apical ends are directed upwards in orientation to light entering obliquely from above, for they remain on the surface in darkness. If the larvae are heavier than the medium, it should be easier for them to swim downward, for the pull of gravity would be added to their ciliary action; consequently, it would be logical to expect that large numbers of larvae kept in darkness and unoriented to light would swim downward and that this response would be universal in the case of larvae illuminated only from below.

A reduction of ciliary action alone does not seem to be an adequate explanation for a positive geotaxis in these larvae; it seems necessary to posit a simultaneous increase in density. Since larvae invariably became geopositive after they had been active for an abnormally long time, it seems probable that their descent to the bottom can be attributed to a hydrostatic principle. The vitelline mass within these organisms may decrease gradually in volume as they grow older and swim longer; and since it is composed largely of fatty globules of low specific gravity, depletion of lipoids and shrinkage of the larvae would decrease their volume and increase their density, thus causing them to descend at the end of the pelagic stage. This view is confirmed by the observation that larvae smaller than average descended to the bottom, whether this condition was attributable merely to normal variations in size or to a visible reduction in volume resulting from an experimentally induced pro-

longation of activity. After prolonged swimming at the surface, larvae were noticeably smaller when they descended to the bottom in solutions of glucose, calcium and hypotonic sea water. In the latter solution it seems probable that they would have swollen somewhat in the beginning and would, therefore, have displaced a greater volume of the medium. They would thus be buoyed up until their volume decreased again through a conversion of food into waste products of metabolism. Since larvae sank passively in hypertonic sea water, they probably lost fluid to the medium, and their subsequent reduction in volume may have made them denser than the solution. Their rapid descent, however, apparently cannot be explained on the principle of osmosis alone. Probably both injury to the cilia and a violent muscular contraction were involved. An initial shock reaction was actually observed under the microscope in larvae exposed to an excess of certain ions; they underwent a violent constriction in the equatorial region, which may have decreased their volume without altering their weight appreciably.

Since du Bois-Reymond (1914) has shown that the same temperature increment will cause a piece of tissue to expand three times as much as a similar volume of water, it seems likely that larvae may sink to the bottom in cold water in a comparatively short time (15–20 min.) because of an increase in density brought about by shrinkage. Similarly the failure of larvae to descend in warm water might be attributed to a swelling of the vitelline mass, which would cause them to displace a greater volume of water; buoyancy would thus hold them just beneath the surface film. Their reaction recalls the behavior of orthotoluidine drops that float on the surface of warm water, but sink to the bottom when the temperature is reduced; like the larvae, these drops, having approximately the same density as water, will rise again when the medium is warmed only a few degrees. What seems to be a downward spiralling movement of the larvae may be merely a succession of circular paths at descending horizontal levels.

#### *Factors influencing metamorphosis*

Only conjectures may be offered at this time regarding the effects of various solutions on the onset of metamorphosis. At first, the writer was inclined to assume that sodium itself is a specific agent that brings about metamorphosis. The rapid setting of larvae in sodium solutions would suggest this hypothesis. Furthermore, sodium would presumably favor the violent muscular contraction necessary for the ejection of the holdfast. The fact that both calcium and magnesium either prevented metamorphosis or delayed it far beyond the normal time of onset might be interpreted on the basis that these ions decreased the permeability of the larval tissues, so that sodium could enter only with difficulty. Concomitance of events, however, suggests but does not prove a causal relationship. In view of the fact that there seem to be many substances capable of inducing metamorphosis in ascidians (Berrill, 1930; Zinkin, 1938), it would not be illogical to predict that the same may be true of bryozoan larvae. If metamorphosis is essentially a process of dedifferentiation, as Huxley (1922) has suggested, it is quite possible that any inhibiting agent can bring it about. Copper is capable of inducing metamorphosis in the oyster (Prytherch, 1934) and has a similar role in ascidian larvae (Grave, 1941; Berthold and Mast, 1944). Miller (1946) reported that copper has some effect in hastening the onset of metamorphosis in bryozoan larvae, but he did not discuss the



experiments that led him to this conclusion. Consequently, it may be that sodium has no specific effect on the larvae of *B. neritina*; it may have either a direct effect by inhibition, and in this respect it would not be unique, or an indirect action by increasing the permeability of larval tissues to ions, such as copper, that have an oligodynamic action. (The effects of ions, especially sodium and copper, on two species from Woods Hole will be discussed more fully in a future paper.)

Caswell Grave (1936) postulated the existence of a by-product of metabolism in ascidian larvae, which is produced in greater quantity by energetic swimming movements. This later product, he says, unites with a "susceptibility factor" formed by the secretion of some larval tissue or organ in the production of a third substance, which apparently acts directly on the larval nerve centers to cause the progressive series of responses that constitute the process of metamorphosis. In *B. neritina*, however, it seems unlikely that the accumulation of metabolic waste products has any influence in hastening metamorphosis. *A posteriori*, it would be more logical to attribute the prolonged activity of larvae under certain conditions to factors that delay the accumulation or concentration of some substance that effects the onset of metamorphosis.

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#### SUMMARY

1. The distribution, external morphology, swimming movements and metamorphosis of the larvae of *B. neritina* are discussed. During metamorphosis the eye-spots of the larva function in orientation to light.

2. At room temperature (26–30° C.) the larvae under laboratory conditions are geonegative and photopositive during the larval period. Just before metamorphosis they lose their intense positive reaction to light and become more or less indifferent.

3. A reduction of temperature causes larvae to become geopositive and simultaneously prolongs their free-swimming period. When either ascending or descending gradients of temperature are employed, the greater number of larvae change their responses to gravity between 20 and 23.5° C.

4. In cold water (7 to 10° C.) larvae lose their intense positive response to light and become more or less indifferent. Their behavior at reduced temperatures shows that they may become geopositive without exhibiting a simultaneous negative reaction to light. Their descent to the bottom of a vial is not caused by a negative phototropism. Either of the two tropisms may be independent of the other.

5. Larvae made geopositive by a reduction in temperature do not swim upwards towards a source of illumination, when the rays can enter a vial only from above. Neither do they react negatively to light, when the rays enter only from below. Their positive geotaxis cannot be modified by light. At room temperature, however, approximately half the larvae, originally geonegative, swim downwards towards a source of light placed beneath a vial.

6. At 28–30° C. the geotaxis of the larvae is not affected by rapid changes in light intensity.

7. At room temperature, intense light (sunlight and artificial illumination) has some effect in driving larvae to the bottom of a vial.

8. When larvae are returned to diffuse daylight after a 30-minute period in darkness, they do not change either their phototropism or their geotaxis, when they are maintained at low (7–8° C.) or high (26–30° C.) temperatures. Darkness, however, probably reduces their activity and shortens the larval period.

9. A reduction (40–50 per cent) of the salt content of sea water greatly prolongs the natatory period and causes larvae to become geonegative after a long period of swimming at the surface. Hypertonic sea water, however, greatly reduces the free-swimming period, induces a more rapid onset of metamorphosis and causes the larvae to become geopositive. The development of larvae that metamorphosed in sea water of various salinities is discussed.

10. A slight increase in the osmotic pressure of diluted (50 per cent) sea water by the addition of a non-electrolyte does not cause the same response as a similar increase in salt content.

11. An excess of either sodium or potassium causes a rapid loss of pigment. Potassium has the more pronounced effect. Sodium induces a rapid onset of metamorphosis, but potassium does not. Both sodium and potassium cause rapid sedimentation of larvae.

12. A similar excess of calcium greatly prolongs swimming movements and inhibits metamorphosis. An excess of magnesium inhibits metamorphosis, but does not cause prolonged swimming.

13. Mechanical agitation, hypotonic sea water and calcium apparently bring about a positive geotaxis by abnormally prolonging the free-swimming period of the larvae.

14. A tentative explanation of geotaxis in these organisms and a discussion of the effects of various factors on their metamorphosis are presented.

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# VARIATIONS OF THE SUBMICROSCOPIC STRUCTURE OF THE CORTICAL LAYER OF FERTILIZED AND PARTHENO- GENETIC SEA URCHIN EGGS

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## INTRODUCTION

In previous papers one of us (A. Monroy and A. Monroy Oddo, 1945, 1946) has investigated by means of polarized light the submicroscopic structure of the cortical layer of the unfertilized sea urchin egg. The cortical layer shows positive birefringence and normal optic axis, and since, in all the experiments made, no data came out suggesting that proteins enter into its constitution, Monroy and Monroy Oddo maintained that it is entirely of lipidic nature. According to its behavior in some experimental conditions, they considered it as a fluid crystal of the smectic type, which can be brought into the scheme of Bungenberg de Jong's complex ionic systems. However, according to Runnström and collaborators (1943-45), whose paper we were able to see only after the publication of Monroy and Monroy Oddo's note, the proteins also take part in the constitution of the cortical layer of both the unfertilized and fertilized sea urchin egg. This question will be discussed in a forthcoming paper.

We deal here with some results obtained by studying the variations of the submicroscopic structure of the cortical layer of the fertilized or parthenogenetically activated egg. Monroy (1945) has already pointed out some rhythmical variations of the cortical birefringence after fertilization, up to the two blastomere stage. We thought it worthwhile to investigate more accurately the rhythms of the observed variations, putting them in relation with the process of mitosis and with the modifications of permeability which can be observed in the fertilized and parthenogenetic egg.

A short note on these findings has already been published in *Nature* (Aug. 17, 1946).

## EXPERIMENTS ON FERTILIZED AND PARTHENOGENETICALLY ACTIVATED EGGS

Observations and experiments were carried on in February-April, 1946, at the Zoological Station, Naples, on eggs of *Psammechinus miliaris* artificially fertilized or activated by Loeb's method. For this purpose, the unfertilized eggs were treated with a solution of 2.8-3.2 cc. of N/10 butyric acid in 50 cc. sea water. After washing in slightly alkaline sea water, they were observed in pure sea water.

During the first series of experiments the temperature of the water was 13°-15° C., in the second series (April), 17°-19°. The developmental cycle was of course remarkably accelerated by the rise of temperature: in the first series the first cleavage of the fertilized eggs and the climax of the first monastral cycle in the parthenogenetic eggs took place after 1 hour and 30 minutes, in the second series after one hour.

*Birefringence of the cortical layer*

**Fertilized eggs.** At fertilization the cortical birefringence of the unfertilized egg disappears, while in polarized light the egg surface acquires a slight diffuse luminosity (Fig. 3). After ca. 15–20 minutes, when the sperm-aster is in course of development, a slight silver-white positive birefringence appears in the cortical layer. In a short time its intensity increases (although it never reaches the intensity it has in the unfertilized egg, and remains always white) and later on it decreases and disappears before the total regression of the sperm-aster. From the very first series of experiments, we noticed a somewhat irregular behavior of this first birefringence cycle; its relation to the sperm-aster cycle was not constant, in some cases its appearance being earlier, in others later than the beginning of the sperm-aster.

In the second series of experiments, which we performed at a higher temperature, the first birefringence cycle was even more irregular. In some lots we could not see it, in others it appeared irregularly and for a short time only in some of the eggs, usually before the sperm-aster was visible. We shall discuss the possible meaning of this behavior later, in connection with the experiments with hypertonic solutions.

A second cycle of cortical birefringence, also with positive character, extends from the metaphase to the telophase of the first mitosis, as already described by Monroy (1945). It is characterized by a much higher intensity of the birefringence, which sometimes reaches the same level as in the unfertilized egg. Furthermore, it is absolutely constant under our experimental conditions.

As soon as the first cleavage is completed, the cortical birefringence disappears. It appears again during the ana-telophase of the second mitotic cycle.

**Parthenogenetic eggs.** In the eggs kept in butyric acid solution, the birefringence becomes more or less white, decreases in intensity and sometimes disappears. After ca. 10 minutes, if the eggs are replaced in sea water, it resumes the intensity and the color of the unfertilized egg, and remains so indefinitely (observations up to one hour after treatment).

The eggs removed from the butyric acid solution after a few minutes and replaced in sea water are activated. A variable percentage, in the various lots, elevate the membrane, while other eggs show incomplete membranogenesis or a total failure of it, depending on individual differences between the females used and on slightly different concentrations or exposure times. The eggs with no membranogenesis, however, are also activated as appears from the nuclear and monastral cycles. It is known in fact, that in the eggs simply activated and not subjected to the second step of Loeb's treatment (hypertony), successive monastral cycles develop, which finally lead to cytolysis.

In the activated egg, with or without a fertilization membrane, the cortical birefringence disappears as in the fertilized eggs. From 15 to 20 minutes later the first birefringence cycle appears and its behavior is entirely similar to that of the fertilized eggs. Also in this case, if the eggs are kept at a higher temperature, the first cycle is more irregular.

The first cycle comes to its end, and, about one hour after activation, the second cycle begins, showing the same characters of constancy and intensity as in fertilized eggs. When the monaster regresses, the cortical birefringence also fades away and

disappears. Half an hour later the second monaster cycle develops and the birefringence reappears. A third, fourth, fifth monaster cycle follows, with more and more accelerated rhythms, and the egg finally cytolyzes. We observed the re-appearance of the birefringence up to the third monaster cycle, and we have little doubt that it appears also in the following.

To summarize, the observations of fertilized and parthenogenetic eggs in polarized light show that in both cases there exists a first cycle of cortical birefringence, rather labile, appearing ca. 15–20 minutes after fertilization or parthenogenetic activation. It soon fades away and the egg shows a slight diffuse luminosity, which is probably due to small granules. Almost synchronic with the maximum expansion of the diaster, or the monaster, the cortical birefringence reappears very intensely. This second cycle also fades away, and a third appears at the metaphase of the second mitosis or at the expansion of the second monaster in parthenogenesis. The birefringent layer is always immediately underlying the hyaline coat of the activated egg.

#### *Experiments with hypertony*

The permeability of the unfertilized, fertilized and parthenogenetic egg has been the object of very many researches and from the experiments of various authors it appears that after activation the permeability undergoes a series of cyclic variations. We will not summarize all the pertinent literature, referring for that purpose to the papers by Herlant (1920), Hobson (1932), Runnström (1923, 1929), Öhman (1945). We will take into account mainly the results of the last two authors. They have shown that the fertilized or parthenogenetic eggs, after treatment with hypertonic saline solutions immediately after activation and for a period of about 10 minutes, manifest a uniform total contraction, preserving an almost unaltered spherical form. To this phenomenon Runnström has given the name of "kugelige Plasmolyse" or spherical plasmolysis. Between 10 and 40 minutes after activation the egg reacts in a different way to hypertonic solution, showing a wrinkled surface, that is, the "eckige Plasmolyse" of Runnström, or angular plasmolysis. After ca. 50 minutes from activation, the spherical plasmolysis reappears, and lasts until the moment immediately preceding the first cleavage when the angular plasmolysis sets in. Runnström (1929) thinks that such phenomena are related to variations of the gelification of the cortical layer, the angular plasmolysis corresponding to a condition of greater rigidity.

We thought that a certain correspondence might exist between the type of plasmolysis and cortical birefringence. Therefore we repeated the experiments of the preceding authors, at the same time examining the eggs in polarized light. We used the same hypertonic solution as Runnström and collaborators, i.e., 20 cc. of sea water and 6 cc. of a 2.5 M solution of NaCl. The eggs were put into the solution at intervals of 5–10 minutes and then observed under normal and polarized light.

The unfertilized egg treated by this solution shows a "polyhedral" plasmolysis (Fig. 2) and loses its cortical birefringence. Immediately after fertilization or parthenogenetic activation, a strong spherical plasmolysis is observed while a superficial hyaline layer becomes visible. It contains small granules and is of variable thickness in different specimens. The underlying cortical layer proper shows a vivid birefringence with polarization cross. Such a condition lasts 10–



15 minutes (experiments made in April, at  $t^{\circ} = 17^{\circ} - 19^{\circ}$ ) and therefore it coincides, in part at least, with the first birefringence cycle observed in normal eggs. After this first period, the angular plasmolysis appears, while in polarized light no cortical birefringence with polarization cross can be detected. The egg surface, especially the edges, appears luminous because of the presence of rather intensely birefringent granules. Such a luminosity always occurs in the cortical layer proper, while the superficial coat, less clearly defined than in the spherical plasmolysis, does not show any luminosity. The angular plasmolysis begins when the evolution of the sperm-aster is in progress.

Such a condition lasts until the metaphase or expanding monaster stage. Then, ca. 50-60 minutes after activation, the eggs in hypertonic solution show again spherical plasmolysis with intense cortical birefringence (Figs. 5, 7, 8). The controls in normal sea water undergo the second birefringence cycle. In this phase the superficial coat in the normal egg has reached its maximum thickness. In eggs treated with hypertonic solution the coat shows an undulated contour, as if, being inelastic, it could not follow the egg mass in its contraction (Fig. 6).

We did not observe a reappearance of the angular plasmolysis shortly before the end of the division, as described by Runnström.

In the parthenogenetic eggs, during the monaster regression the angular plasmolysis reappears, while in the controls birefringence disappears. In the second, third, etc., monaster cycle the spherical plasmolysis reappears, always in association with birefringence.

To summarize: these experiments show that the two types of plasmolysis mentioned by the authors quoted above and observed again by us, correspond fairly exactly with the cycles of cortical birefringence; namely, spherical plasmolysis corresponds with the phases of evident cortical birefringence, angular plasmolysis corresponds with the phases of lacking birefringence. We should like to point out again the fact, which will be discussed later, that during the first 10-15 minutes after activation, the cortical birefringence of the controls, at the conditions of temperature stated above, does not always manifest itself clearly. The hypertonic treatment, on the contrary, makes it very striking in all the eggs.

We also noticed that hypertony makes more evident a birefringence of the asters, already described by Schmidt (1939) and Monné (1945) (Figs. 5, 7, 8).

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#### PLATE I

All the figures are photographs of *Psammechinus miliaris* eggs at a magnification of 500  $\times$ .

FIGURE 1. Unfertilized egg (polarized light): note the cortical birefringence.

FIGURE 2. Unfertilized egg in hypertonic sea water (ordinary light): note the "polyhedric" plasmolysis.

FIGURE 3. An egg soon after fertilization (polarized light): note the birefringence of the fertilization membrane and the disappearance of the cortical birefringence.

FIGURE 4. An egg in the late metaphase stage (polarized light): the birefringence of the cortical layer is again evident.

FIGURES 5 AND 6. An egg in the late metaphase stage in hypertonic sea water as seen in polarized light (Fig. 5) and in ordinary light (Fig. 6). Note the smooth surface of the egg exhibiting a very brilliant birefringence (Fig. 5) and the wrinkled hyaline layer which does not exhibit any birefringence (Fig. 6). Note also the birefringence of the spindle as seen from a pole.

FIGURES 7 AND 8. Eggs in the beginning (Fig. 7) and late telophase (Fig. 8) in hypertonic sea water (polarized light): birefringence of the cortical layer and of the spindle-figure.

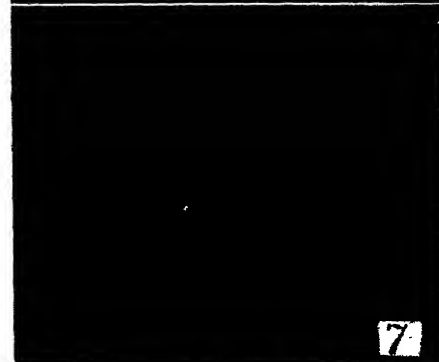
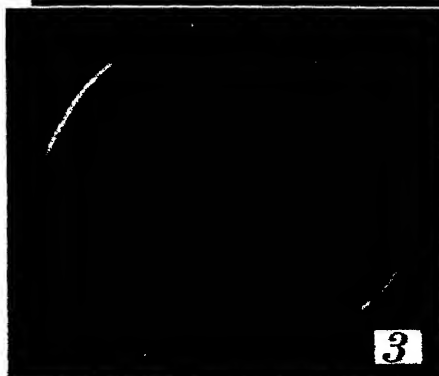
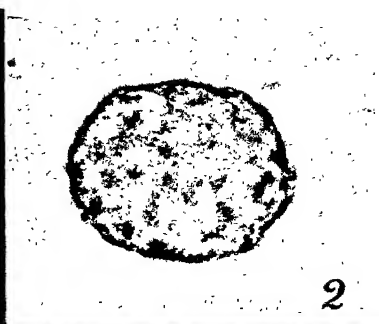


PLATE I

*Colchicine experiments*

The synchronism of the birefringence and the diaster and monaster cycles (in fertilized and parthenogenetic eggs respectively) as well as of the plasmolysis cycles leads one to think that all these phenomena are linked with one another. We were able to observe, however, that the first birefringence cycle (15–20 minutes after

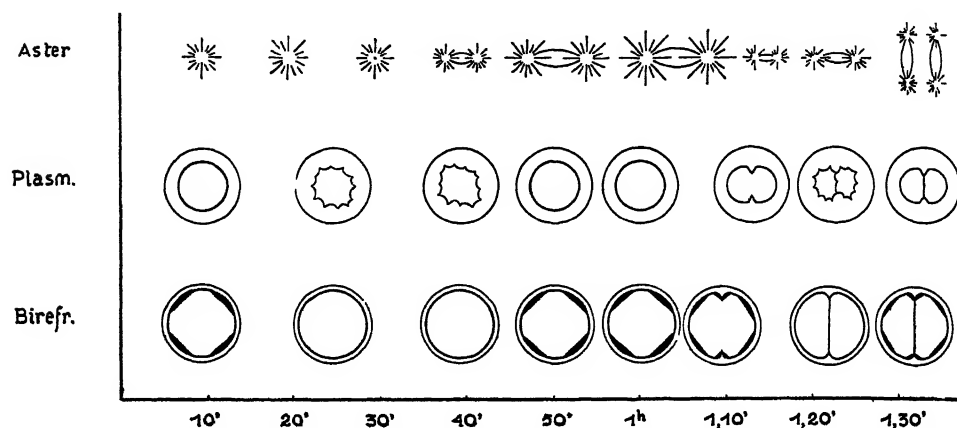


FIGURE 9. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of sperm-aster and spindle and the types of plasmolysis in fertilized eggs.

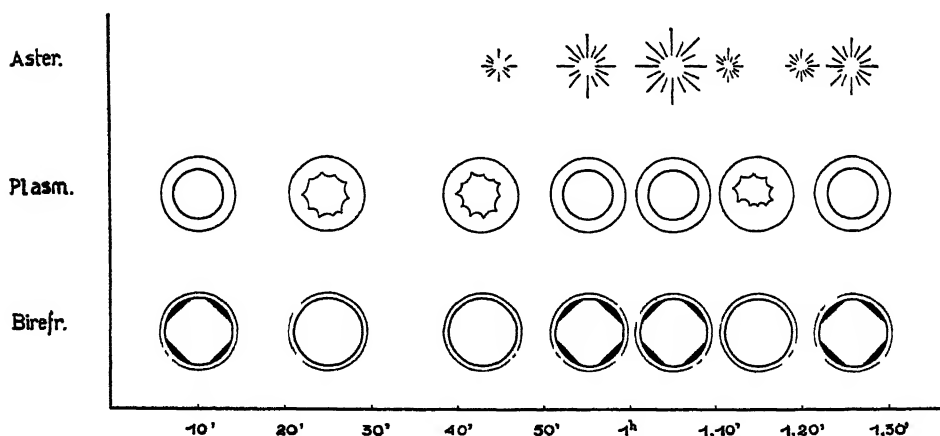


FIGURE 10. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of monaster and the types of plasmolysis in eggs activated with butyric acid.

fertilization) is not strictly correlated with the development of the sperm-aster, and in the parthenogenetic eggs, of course, is not connected with any phenomenon comparable to it. In order to test any possible correlation between the cortical birefringence and the aster cycles, we have treated the eggs with colchicine, with the aim of suppressing the aster.

The technique was the same as that used by Beams and Evans (1940), i.e., immersion of the fertilized or parthenogenetic eggs in a 0.0002 M solution of colchicine. In both series of experiments we observed the birefringence cycles, as already described, in complete absence of asters. The results are extremely clear cut, and therefore we can conclude that the cycles of the cortical birefringence, although normally synchronous with the cycles of the asters, are independent of them.

Furthermore the cycle of the two types of plasmolysis in colchicine-treated eggs proceeds exactly in the same way as in the controls. It is thus clear that the suppression of the aster by colchicine does not alter in any way the conditions of permeability of the cortical layer. Hence these experiments confirm Runnström's idea that the two types of plasmolysis are related to different conditions of the cortical layer only and not of the whole cytoplasm.

### CONCLUSIONS AND DISCUSSION

From the above described investigations the following conclusions can be drawn:

1. The cortical layer of the unfertilized egg of *Psammechinus miliaris* shows a positive birefringence of yellow-orange color (Fig. 1).
2. Such birefringence disappears at fertilization or parthenogenetic activation (Fig. 3).
3. From 15 to 20 minutes after fertilization or activation, in certain conditions, a slight positive birefringence appears, of silver-white color. Hypertonic treatment makes it evident and constant also in cases in which in the controls it is faint or absent.
4. From 20 minutes to the end of metaphase, the cortical layer does not show any birefringence either in controls or in hypertonic treated eggs.
5. Beginning with the end of metaphase in fertilized eggs and with the starting of the monaster expansion in parthenogenetic eggs, a strong positive cortical birefringence appears, silver-white in color and very brilliant both in controls and in hypertonic treated eggs (Figs. 4, 5, 7, 8).
6. These cycles of variation of the submicroscopic structure of the cortical layer correspond exactly to the cycles of spherical and angular plasmolysis described by Runnström.
7. Aster inhibition by colchicine does not alter the cycle either of the cortical birefringence or of plasmolysis. The correspondence of the cycles of cortical birefringence, of sperm-aster and spindle in fertilized eggs, of monaster in parthenogenetic eggs and of the type of plasmolysis is summarized in the diagrams (Figs. 9, 10).

It is thus evident that after fertilization the cortical layer of the sea urchin egg undergoes cyclic variations of its submicroscopic structure, which are revealed by polarized light. Runnström, Monné and Broman (1943), on the contrary, did not find any variations of the cortical birefringence after fertilization, although they were surprised by the fact that corresponding to the modifications observed by Runnström (1923, 1928) in dark field, no analogous variations could be detected in polarized light.

We have already stated that the cortical birefringence which reappears soon after fertilization is very variable in intensity and constancy in the different lots and under different experimental conditions. Hypertonic treatment, however, makes it clear and constant in every egg. Furthermore we have noticed that in some eggs, a few minutes after fertilization (4–5 minutes) and, even more frequently, after parthenogenetic treatment, a very labile and short lasting birefringence appears, which soon fades away. All this indicates, to our opinion, that the cortical layer, after normal or parthenogenetic activation, up to ca. 15–20 minutes, is in a condition of particular lability in its submicroscopic structure, which probably has a physiological correspondence in the peculiar susceptibility to hypotonic solutions pointed out by Just (1922). Since the hypertonic treatment makes the birefringence evident, it can be assumed that, as an effect of activation, at first there is an increase of the distance between the molecules of the cortical layer, which however do not lose their orientation. Hypertony, by causing a decrease of volume, determines the approach of the molecules, thus showing their orientation.

We are not able, at the moment, to give an interpretation of the intimate mechanism of such morphological modifications, but we want to draw attention to the remarkable physico-chemical modifications which, as is well known, occur soon after fertilization. We regard it as probable that an important feature possibly concerned with the molecular orientation of the cortical layer, should be the variation of the distribution of some electrolytes and particularly of Ca, which have been observed by many authors soon after fertilization, e.g., Mazia (1937), Örström and Örström (1942), A. Monroy Oddo (in press). In fact, the importance of bivalent electrolytes (especially Ca) on the molecular orientation and attraction on complex ionic systems is known (Bungenberg de Jong et al., 1935; 1938). A loss of Ca may thus determine a decrease of the effective attraction between the molecules in the ionic layer, and thus a condition of greater hydration of the whole system. In this connection it is also interesting to note Öhman's (1945) observation that after fertilization a decrease of the free cephalin occurs, which, according to the author, is becoming linked to the proteins. That means a variation of the relations between the various constituents of the system, and it is not improbable that this fact also plays a rôle in determining a rearrangement of the composition and structure of the cortical layer.

Attention should also be called to a fact which might have some importance for the analysis of the action of butyric acid in the activation process. Unfertilized eggs put into butyric acid show a decrease in the intensity of the cortical birefringence, which at the same time becomes whitish. After a certain time in the acid the birefringence returns to the same condition as in unfertilized eggs. The period of decreased intensity corresponds approximately to the optimum time for a successful activation.

Following the first phase the second succeeds, lasting from 15–20 minutes up to 50–60 minutes after activation, and during this phase the birefringence disappears and angular plasmolysis sets in. One can interpret this fact by assuming that after the phase of molecular orientation characteristic of the unfertilized and freshly fertilized egg, a phase of disorientation follows. At this time the egg appears diffusely luminous in polarized light, and this is probably due to the lack of normality of the optic axis of the cortical layer.

As to the correspondence which we found between birefringence and spherical plasmolysis, and respectively isotropy and angular plasmolysis, we are inclined to think that in the first case the cortical layer, being formed by radially iso-oriented molecules, undergoes uniform contraction due to hypertony, while in the second case, because of the disordered molecular orientation, the resistance of the various points of the cortical layer varies, and thus it reacts to plasmolysis not by a uniform contraction, but by formation of wrinkles (cf., also, Runnström, Monné and Broman, 1943).

It might seem strange that the reaction of the fertilized egg to hypertony, while its cortical layer has an orientated structure, is different from the reaction of the unfertilized egg, where the cortical layer also has an orientated structure apparently entirely similar. The unfertilized egg in fact, as we said, in hypertonic solution contracts with a "polyhedric" surface and loses its cortical birefringence. We have already mentioned the chemical and structural variations which probably occur in the cortical layer of the egg after fertilization and in particular the loss of Ca and perhaps also of some of its lipidic components (cephalin?). This results in a condition of greater softness than in the unfertilized egg. In the latter, the Ca content of the whole egg and probably also that of the cortical layer is greater, and this causes a greater condensation of its molecules. This is probably the cause of a higher rigidity upon which the characteristic type of plasmolysis depends. It is not improbable, however, that in this phenomenon a rôle is played also by particular relations of the molecules of the cortical layer with the subcortical proteins, as the researches of Runnström, Monné and Broman (1943) seem to suggest. They found that trypsin-treated unfertilized eggs in hypertonic solution contract with a smooth surface, without losing their cortical birefringence.

Apparently this mechanical interpretation fits the observed facts better than Öhman's (1945) explanation. He considers the cortical layer as a lipo-proteic film, in which the quantity of proteins and lipids may vary. Of course our interpretation does not exclude the possibility that during the two phases, viz., orientation and disorientation, the relations between cortical lipids and subcortical proteins may also vary. Herlant's (1920) observations on the variations of susceptibility of the egg to fat solvents which occur between fertilization and first cleavage do not seem to coincide well with the rhythms of plasmolysis; they seem rather to indicate the existence of phases of greater and lesser susceptibility which are not easy to interpret because of their irregularity. At any rate, it would probably be worthwhile to repeat such experiments in connection with observations in polarized light, taking into account especially Bungenberg de Jong's researches on the mode of action of organic and inorganic compounds on complex ionic systems, and in particular on the influence they have on the length of the carbon chain.

It is perhaps pertinent to recall here an observation by Öhman (1945), viz., that the formation of bubbles by the action of heat is entirely inhibited soon after fertilization i.e., during the contraction phase. Later on the frequency with which eggs show bubbles gradually increases and reaches a maximum between 20 and 30 minutes after fertilization. Then another phase sets in, characterized by a lesser frequency of bubble formation (not total inhibition as soon after fertilization) which extends up to the anaphase of the first mitosis, at which time it increases again. The coincidence of this rhythm with the phases of cortical orientation and disorienta-

tion which we have described is very striking. The fact might possibly mean that the bubble formation is greatly facilitated by the molecular orientation of the cortical layer, as was to be expected on the ground of Bungenberg de Jong and Bonner's (1935) researches on bubble formation in drops of coacervates.

In conclusion we believe that the lipidic character of the cortical layer of the egg is not lost at fertilization, although rearrangements in its chemical composition and consequent variations of physical properties may well take place. Furthermore we recall that one of us (Monroy, 1945) has already pointed out that the cortical birefringence which reappears after fertilization, during the second cycle, has the same characters and the same quantitative value as in the unfertilized egg.

Furthermore the following fact found by us seems interesting: while birefringence conditions and type of plasmolysis are undoubtedly linked to one another, no linkage seems to exist between these phenomena and the aster cycles, although some synchronism is undeniable. The colchicine experiments allowed us to demonstrate that the birefringence-permeability cycle can proceed entirely undisturbed when asters are inhibited. It is also a remarkable fact that in parthenogenetically activated eggs, at the end of the monaster cycle, angular plasmolysis reappears as in fertilized eggs at the end of cleavage. Clearly enough we are dealing here with a rhythm characteristic of the activated egg independent of cellular division.

Our researches have not thrown light on the submicroscopic structure of the superficial hyaline layer, which, as is well known, appears after fertilization and is more evident in hypertony. It did not show any birefringence in our experimental conditions. We were able to observe that the cortical birefringent layer is always underlying the hyaline coat (as already stated by Runnström, Monné and Broman, 1943) and that the latter, especially during the second phase of spherical plasmolysis, is apparently non-elastic, and therefore, being unable to follow the egg in its contraction, shows wrinkles and undulations. This observation seems to be in favour of the presence of some sort of cleavage between the hyaline coat and the cortical layer, and that would explain how E. B. Harvey (1934) was able to shift it entirely from the egg by means of centrifugation.

#### SUMMARY

The authors have investigated the structural variations of the cortical layer of *Psammochinus miliaris* egg after fertilization or parthenogenetic activation, by means of polarized light, hypertonic treatment and colchicine.

They found regular cyclic variations of the birefringence of the cortical layer. A first inconstant cycle of birefringence appears at 15-20 minutes after fertilization or parthenogenetic activation. A second, more intense and constant cycle appears at the end of metaphase up to the telophase of the first cleavage, or, in parthenogenetic eggs, at the expansion of the monaster.

In eggs treated with hypertonic solution, "spherical plasmolysis" corresponds to the birefringent phases, while "angular plasmolysis" corresponds to the non-birefringent phases.

Colchicine, which inhibits the aster formation, does not alter either the birefringence or the plasmolysis cycles and their synchronism. The latter two phenomena are thus linked to one another, but independent of the cycles of the aster.

The meaning of the facts and their relations to the physico-chemical phenomena occurring during activation are discussed.

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# PHYSIOLOGICAL OBSERVATIONS UPON A LARVAL EUSTRONGYLIDES. XI. INFLUENCE OF OXYGEN TENSION ON THE AEROBIC AND POST-ANAEROBIC OXYGEN CONSUMPTION.

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Harnisch (1932a, b; 1933; 1935a, b, c) has emphasized that the respiration of parasitic worms is dependent on the oxygen tension at least up to a tension of 760 mm. Hg. He has also shown that the post-anaerobic oxygen consumption, the repayment of an oxygen debt, shows the same relationship in a number of free living organisms, the normal aerobic respiration of which is much more independent on the tension. He has used this argument as one of the cornerstones for his hypothesis that the respiration of parasitic worms and the post-anaerobic oxygen consumption of free living animals is governed by another set of enzymes (secondary aerobiosis) than the normal aerobic respiration (primary aerobiosis) of the latter. Von Buddenbrock (1939) has questioned this view in so far as free living animals are concerned, pointing out that the tension at which the oxygen consumption begins to decline is not static but changes with the intensity of the oxygen consumption.

Since Harnisch used for his investigations on parasites only intestinal and bile-duct helminths, it appeared worthwhile to study in this respect the larvae of *Eustrongylides ignotus* which definitely lead in nature a much more aerobic life than the former (von Brand, 1942). It was hoped to decide between Harnisch' and von Buddenbrock's views by using larvae at various levels of metabolism, the changes being partly induced by the stimulating effect of the potassium ion (von Brand, 1943), partly by various temperatures. Although this hope was not completely fulfilled, the experiments in themselves are of sufficient interest to warrant publication.

## MATERIAL AND METHODS

In the respiration experiments reported upon previously in this series (von Brand, 1942, 1943) *Eustrongylides* larvae freshly isolated from the cysts which they normally occupy in *Fundulus* were used almost exclusively. It was then found that they showed initially a trace of excess oxygen consumption. This phase had to be eliminated for the present experiments. The worms were therefore isolated in batches from 3 to 5 specimens, weighed, and kept over night in a shallow layer of either 0.85 per cent NaCl or 1.14 per cent KCl, that is, under conditions allowing for a completely aerobic metabolism. After this preliminary period their oxygen consumption was studied at various oxygen tensions with a minimum of 1½ hour for each tension. The fluid in the respiration vessels was the same as that used during

<sup>1</sup> The author is indebted to the Elizabeth Thompson Science Fund for a grant towards the purchase of the respiration apparatus used in this investigation.

the preliminary period. The oxygen determinations were carried out by means of a Warburg apparatus. The gas mixtures were passed for 20 minutes through the vessels before closing the manometers; they contained oxygen at the following tensions: 760, 160, 34, and 6 mm. Hg. Usually from 2 to 3 different tensions were used consecutively with one batch of worms. Care was taken to employ the highest tension first in order to avoid an excessively high oxygen consumption if the worms should acquire an oxygen debt at the lower tensions. Previous experiments had shown that the respiration of the larvae remains virtually constant over long periods if the conditions are not changed. Any decline in oxygen consumption following the replacement of a gas mixture with high oxygen tension by one containing less oxygen could consequently safely be ascribed to the lowered tension. The experiments of these series were carried out at 40.2, 28.5, and 18.8° C.

A slightly different procedure had to be employed in the series in which the dependency of the post-anaerobic oxygen consumption on the tension was studied. The isolated and weighed worms were first exposed, in 0.85 per cent NaCl, for a period of 16 hours to anaerobic conditions, using the method previously described (von Brand, 1942). In order to get as uniform an oxygen debt as possible, the worms were subjected to this anoxic period at 37° C. regardless of the temperature at which the post-anaerobic oxygen consumption was studied subsequently. The repayment of an oxygen debt is a transitory phenomenon; after an initial high rate, the oxygen consumption declines slowly until it reaches finally the pre-anaerobic level. The phase of high oxygen consumption will last longer at low than at high temperatures if an equal oxygen debt is repayed at both temperatures. These facts precluded obviously the consecutive use of various gas mixtures with one and the same group of animals. Instead, a new batch of worms was used for each experiment at each tension and only the oxygen consumed during the first half hour was taken into consideration. These experiments were carried out at the same tensions and temperatures as those mentioned above; an additional series was performed at 9.8° C.

## RESULTS AND DISCUSSION

A summary of the experiments is presented in Table I. It is evident that the dependency of the oxygen consumption on the tension is much more pronounced at high than at low temperatures if the comparison is made between series performed under otherwise equal conditions. In the aerobic NaCl series, for example, the oxygen consumption was dependent, at 40.2° C., on the tension over the entire range of tensions studied, while at 18.8° C., it was almost constant in the range of 760 to 34 mm. Hg.

The post-anaerobic oxygen consumption was, at each temperature studied, about three times as high as the normal aerobic respiration in NaCl solution when the values found at an oxygen tension of 760 mm. Hg are compared.

If, as von Buddenbrock (1939) maintains, the intensity of the respiratory rate decides at what tension the consumption begins to decline, one should expect that at each temperature the curve for the post-anaerobic oxygen consumption should be steeper than that of either the aerobic NaCl or KCl series. The observations confirm, on the whole, this view, especially at tensions below 160 mm. Hg. Between 760 and 160 mm. Hg there was hardly a significant difference between the three

TABLE I  
*Influence of oxygen tension on the normal aerobic and the post-anaerobic oxygen consumption of larval Eustrongylides ignotus.*  
*Under oxygen consumption the mean values with the standard error of the means and, in brackets, the extremes are listed*

Oxygen tension, mm. Hg	Temp. °C.	Aerobic, 0.85 per cent NaCl				Aerobic, 1.14 per cent KCl				Post-anaerobic, 0.85 per cent NaCl			
		760	160	34	6	760	160	34	6	760	160	34	6
Experiments, No.	40.2	14	19	12	8	7	15	8	12	8	8	8	8
O <sub>2</sub> consumption mm. <sup>3</sup> /g./half hour		139±11 (75, 216)	104±5 (69, 140)	87±4 (62, 110)	27±2.2 (19, 36)	174±9 (137, 214)	148±9 (100, 214)	100±9 (62, 149)	22±2.8 (11, 37)	373±7 (349, 412)	318±25 (246, 468)	131±15 (68, 204)	38±4.8 (16, 58)
Experiments, No.	28.5	16	24	14	20	15	16	15	15	12	12	12	10
O <sub>2</sub> consumption mm. <sup>3</sup> /g./half hour		67±3.3 (43, 88)	51±3.2 (31, 84)	48±3.9 (30, 73)	32±2.5 (10, 52)	86±3.7 (63, 107)	69±4.5 (39, 117)	70±3.0 (53, 89)	27±3.0 (10, 51)	196±5 (164, 224)	177±6 (161, 215)	91±7.7 (42, 132)	30±4.4 (9, 47)
Experiments, No.	18.8	12	12	12	12	12	12	12	12	11	12	12	11
O <sub>2</sub> consumption mm. <sup>3</sup> /g./half hour		30±1.9 (16, 39)	29±2.3 (19, 42)	28±1.7 (19, 40)	13±0.8 (8, 20)	41±2.5 (25, 54)	38±1.7 (29, 48)	37±2.9 (22, 54)	13±2.1 (5, 28)	87±2.9 (76, 101)	83±3.6 (71, 97)	61±2.3 (48, 72)	12±2.0 (5, 23)
Experiments, No.	9.8									10	12	12	12
O <sub>2</sub> consumption mm. <sup>3</sup> /g./half hour										46±1.9 (38, 57)	43±1.9 (33, 52)	39±1.5 (27, 48)	11±1.2 (4, 18)

conditions studied, with the exception of the 40.2° series which showed the postulated relationship. To make von Buddenbrock's view of the essential similarity between normal and post-anaerobic oxygen consumption entirely acceptable, another prerequisite would have to be met. One should expect that the dependency of the post-anaerobic oxygen consumption on the tension should be the same as the aerobic one if one compares series in which the intensity was equal at a tension of 760 mm. Hg, i.e., if one takes as basis of comparison post-anaerobic experiments performed at lower temperatures than the aerobic ones. The post-anaerobic experiments performed at 18.8° C. showed at 760 mm. Hg exactly the same intensity in oxygen consumption as the 28.5° C. KCl series. Similarly, the rate of oxygen consumption in the 9.8° C. post-anaerobic series was quite similar to that found in the 18.8° C. KCl series. In both cases a slightly greater dependency on the tension was observed in the post-anaerobic series; the differences are, however, not statistically significant.

While the present experiments seem, therefore, to support von Buddenbrock's view, they do not necessarily as yet completely disprove Harnisch's conception of "primary" and "secondary aerobiosis." Harnisch has found evidence that the respiration of parasitic worms is almost exclusively of the second type, that is, it corresponds to the post-anaerobic oxygen consumption of free living animals. One would then also expect identical curves in cases where the metabolic levels coincide. Although the larvae of *Eustrongylides ignotus* resemble in their general metabolism unquestionably free living animals more than they do intestinal helminths, it cannot be stated categorically that they do so in the question under consideration. To decide definitely between Harnisch and von Buddenbrock, experiments similar to those described in the present paper should be performed on free living animals. Certainly, von Buddenbrock's criticism of Harnisch's failure to consider the intensity factor is justified, but that is hardly sufficient to reject the latter's views without further experimentation.

One further point deserves brief discussion. Von Buddenbrock (1939) has pointed out that the reasons for assuming a dependency of the oxygen consumption on the tension are occasionally rather insecure because the gases going into purely physical solution are neglected. He cites the example of the sea urchin *Sphaerechinus granularis* which shows for a certain period an increased oxygen consumption if it is transferred from sea water with normal oxygen content into water containing an abnormally high oxygen content. Analyses of the coelomic fluid revealed that the greatest part of this excess oxygen had simply gone into physical solution and had not participated in the metabolic processes. Such an explanation cannot be applied to the *Eustrongylides* larvae. Some of the worms were, after their metabolic level had been ascertained at an oxygen tension of 160 mm. Hg, kept up to 6 hours in pure oxygen at 40.2° C. They showed during the entire time a regular oxygen consumption which was all the time higher than that found in the preliminary period. Obviously, we are dealing here with a case of true dependency on the tension, just as it occurs in actinians which are, as is known since Henze's (1909) investigation, a classical example for this type of relationship.

It should finally be noted that the exposure to pure oxygen for 6 hours did not seem to harm the *Eustrongylides* larvae. They differ in this respect from the intestinal helminth *Ascaris* which is rapidly killed by oxygen of high tension (Laser,

1944). This difference may have a biological basis in the fact that the latter worm lives normally in a much oxygen poorer habitat than the former.

### SUMMARY

1. The oxygen consumption of larval *Eustrongylides ignotus* shows a greater dependency on the oxygen tension at high than at low temperature.

2. The post-anaerobic oxygen consumption is more dependent on the tension than the respiration of larvae that were exposed previously to well oxygenated surroundings, if experiments performed at equal temperatures are compared. This difference disappears almost completely, however, if experiments are compared in which the intensity of the oxygen consumption was equal in both sets at a tension of 760 mm. Hg.

3. The implication of these data is discussed on the controversy between Harnisch and von Buddenbrock as to whether it is justified to distinguish between "primary" and "secondary aerobiosis."

4. The dependency of the oxygen consumption on the tension is a true one and is not only simulated by oxygen going into physical solution in the body fluids at higher tensions.

5. The larvae of *Eustrongylides*, in contrast to *Ascaris*, are not killed by a 6-hour exposure to pure oxygen.

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# THE BIOLOGICAL BULLETIN

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## RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX I

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There have been two prime viewpoints regarding the relation of metabolism to morphogenesis. One advanced by Child and his students has considered rate of metabolism in relation to the organismal factors initiating and directing the course of morphogenesis. Barth (1938, 1940a, 1940b), supporting Child's view, has suggested that increased oxygen tension in the tissues adjacent to a wound may furnish a primary stimulus for regeneration in hydroids; but he found that rate of regeneration was much more markedly stimulated by high oxygen tension than was rate of oxygen consumption. Thus the effect on regeneration may not have been mediated through an effect on total aerobic metabolism. The other viewpoint, advanced by Tangl (1909) and more recently by Tyler (1933, 1936, 1942), has considered rate of metabolism in relation to the release and utilization of energy in morphogenetic processes, particularly differentiation. Tyler concluded from his work on sea urchin embryos that differentiation requires the expenditure of metabolically released energy in addition to that required for maintenance, and that this energy does not become resident in structure but is released as heat during morphogenesis. This view is consistent with the work on "activity metabolism" (cf., Fisher et al., 1942, 1944).

The present study was undertaken to analyze further the relations between metabolism and morphogenesis from both viewpoints. In the regenerating annelid, measurements of morphogenesis may be made by counting the number of new segments produced (Stone, 1932 and Coldwater, 1933). The formation of a new segment is not a simple event, but involves distinct stages. This allows estimation of the rates at which various processes in regeneration proceed. Metabolism may be separated into fractions by means of poisons, and the relation of the activity of particular cellular respiratory systems to morphogenesis may then be tested. Two modes of approach were used in this study: oxygen consumption and respiratory sensitivity to poisons were measured at various times during regeneration (results reported in this paper), and the effects of continuous poisoning and of high and low oxygen tension were determined by measuring the progress of regeneration (results to be reported later).

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## MATERIALS AND METHODS

Worms of the species *Tubifex tubifex*<sup>1</sup> were rigorously selected for uniform size and condition (3.5 to 5.0 centimeters in length, a light, smoothly graded color, no signs of previous regeneration, no signs of breeding condition). For a week before use and between determinations the worms were kept in frequently changed tapwater at about 17°C. A 0.2 per cent solution of chloretone was used as an anaesthetic during amputation of the posterior two-fifths of each worm and during examination under the microscope. Individual worms were kept in anaesthesia no longer than ten minutes every third day (cf., Stone, 1932). Stage and rate of regeneration were estimated at intervals of several days. Three stages in the formation of a new segment were clearly distinguishable *in vivo*. First the new segment appeared as a narrow shadow perpendicular to the longitudinal axis of the worm. Later the new segment was slightly longer and the septum appeared as a pair of fine sharp lines. Finally the new setae were visible as small refractile bodies. The three stages were considered to be stages of localization, early differentiation, and later differentiation respectively (cf., Stone's description of the cytology and histology of regeneration in *Tubifex tubifex*). The number of segments in each of the stages was recorded separately. After regeneration had proceeded for a week or more, all stages of segments were present in the new tail, but segments which were hard to classify were few in number. "Rate of localization"<sup>2</sup> was calculated as the increase in total number of segments per worm per day; "rate of early differentiation" as the increase in number of segments in all stages of differentiation, and "rate of later differentiation" as the increase in number of segments with setae.

Most of the determinations of oxygen consumption were made by the Warburg manometric method, at 25°C. Worms for these determinations were taken in groups of twenty-five to forty, collected into a ball, rolled on filter paper to free them of excess water, placed in a pan of platinum foil and weighed quickly on a torsion balance. Consecutive weighings were found to deviate by less than one per cent. A difficulty in use of intact animals in the Warburg apparatus was encountered in the differences in degree of dispersion of the worms over the bottom of each flask. Probably the marked ability of *T. tubifex* to contract "oxygen debt" (Harnisch, 1935) was brought into play when the worms remained in a ball (average oxygen consumption in five determinations during which the worms remained in a ball was 0.12 milliliter per gram wet weight per hour compared to 0.16 milliliter for dispersed worms). Fortunately the worms spread out uniformly in most cases and only those determinations throughout which the worms were uniformly dispersed were included when calculating averages.

Successive determinations were made on the same worms at intervals of several days and the progress of regeneration was also measured. That this routine was not unduly injurious to the worms was indicated by a normal rate of regeneration and a casualty rate only slightly higher than that of worms upon which no manometric determinations were made; also the oxygen consumption of intact worms used as control remained constant within the limits of error throughout each series of

<sup>1</sup> Identification confirmed by Dr. R. G. Stone, University of Kansas City.

<sup>2</sup> This is the same as rate of regeneration as used by Stone (1932) and Coldwater (1933), although these authors did not calculate rate on a daily basis. From their published data on comparable worms the curve in Graph 1 may be duplicated.

determinations. These intact worms were anaesthetized and examined as were the experimental animals, injured individuals being removed from the group. About 10 per cent of the worms in each group had been removed by the end of the experiments.

A modification of the method reported by Howland and Bernstein (1931) was used for measuring oxygen consumption by individual worms. Each worm was drawn into a capillary tube 0.5 millimeters in diameter. By shifting the worm in a drop of water back and forth in the tube, air was drawn in at each end, then a drop of 5 per cent potassium hydroxide solution. Finally one end was sealed with paraffin oil and the other with vaseline (see Fig. 1). The tube was mounted in a

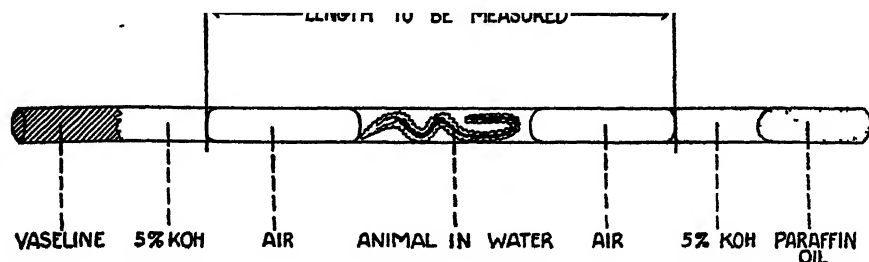


FIGURE. 1 Diagram of respirometer used in modified Howland-Bernstein method of measuring oxygen consumption (much enlarged).

vertical position on a glass plate in a glass-walled constant-temperature bath at 21°C. About fifteen such tubes were handled at a time. The distance between upper and lower air-KOH interfaces was measured using a horizontal microscope with a calibrated adjustment. The inside diameter of the capillary was measured exactly and the volume of oxygen consumed was calculated directly. A wormless tube served as a thermobarometer. The movement of the worm in the tube was found to furnish an efficient stirring mechanism in the fluid part of the system, and it was calculated that the capillary was not fine enough to hinder diffusion in the gaseous phase. After a series of readings, the capillary tubes were broken at the air spaces and the worms removed uninjured. Measurement of each worm was then carried out by anaesthetizing it and taking the diameter at four or five points and the length. The volume of the worm was computed as a series of cylinders.

#### DATA ON WORMS NOT UNDERGOING REGENERATION

As determined both by use of the Warburg apparatus and by the method just described, the rate of oxygen consumption of normal worms was within the range of rates reported by other workers using various methods (Dausend, 1931; Harnisch, 1935; Brazda and Rice, 1940). In agreement with the last named investigators, it was found that size of worms from 2.5 to 5.0 centimeters in length made no significant difference in rate of oxygen consumption. Concentrations of potassium cyanide from  $2 \times 10^{-6}$  M to  $2 \times 10^{-5}$  M had no inhibitory effect on oxygen consumption by normal worms (see Table I). Cyanide in a concentration of  $2 \times 10^{-2}$  M was rapidly lethal, but nevertheless did not decrease oxygen consump-



tion as drastically as its toxicity might suggest. Comparison with the data reviewed by Commoner (1940) shows that the total oxygen consumption by *T. tubifex* is of the same absolute magnitude as the cyanide-stable fraction of respiration in many other animals and tissues for which such data are available.

TABLE I  
*Oxygen consumption by intact worms in various concentrations of potassium cyanide*

Solution	$O_{O_2}$	Number of determinations
Tapwater	0.16	22
$2 \times 10^{-6}$ M KCN	0.16	2
$2 \times 10^{-5}$ M KCN	0.16	6
$2 \times 10^{-4}$ M KCN (non-lethal)	0.15	5
$2 \times 10^{-3}$ M KCN (semi-lethal)	0.19	2
$2 \times 10^{-2}$ M KCN (lethal)	0.11	1

Note: Determinations were made using standard manometric procedure at 25°C.

Respiratory quotient was found to average 0.68 for normal starving worms. The data agree with those of Brazda and Rice and suggest non-carbohydrate metabolism, possibly protein or fat metabolism. Such a type of metabolism is also expected from the cyanide-stable character of the respiration (cf., Commoner's review) as well as from the starving condition of the worms.

#### EXPERIMENTAL RESULTS

In preliminary work measuring the oxygen consumption of individual worms (see Table II) it was found that during the first few days of regeneration oxygen

TABLE II  
*Oxygen consumption by individual worms*

Condition	Oxygen in mm. <sup>3</sup> per mm. <sup>3</sup> of worm per hour					
	0 days	1 day	8 days	11 days	15 days	19 days
Normal intact	0.10	0.10	0.11	0.09	0.09	0.09
Normal cut (regenerating)	0.10	0.12	0.14	0.17	0.15	0.15
X-rayed intact	0.08	0.09	0.07	0.09	0.08	0.11
X-rayed cut (inhibited)	0.10	0.11	0.09	0.11	0.10	0.11

Note: Determinations were made using capillary tubes at 21°C. Probable error was  $\pm 0.01$ .

consumption proceeds at a rate only slightly higher than normal. Later a marked increase in rate of oxygen consumption appeared: 0.08 mm.<sup>3</sup> per mm.<sup>3</sup> of worm per hour, or 85 per cent above normal. Subsequently the rate of oxygen consumption decreased. It was found that worms in which regeneration had been inhibited

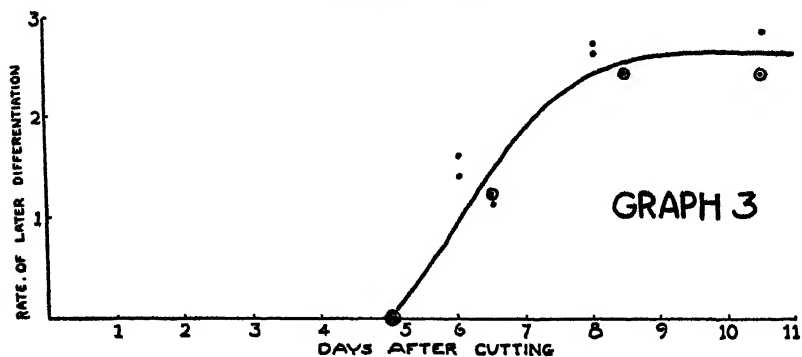
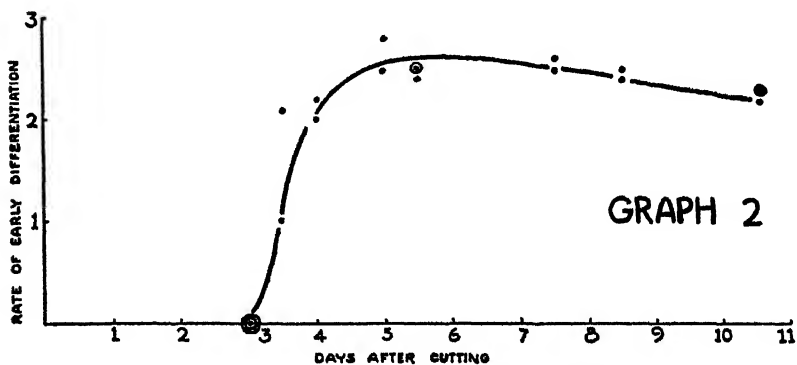
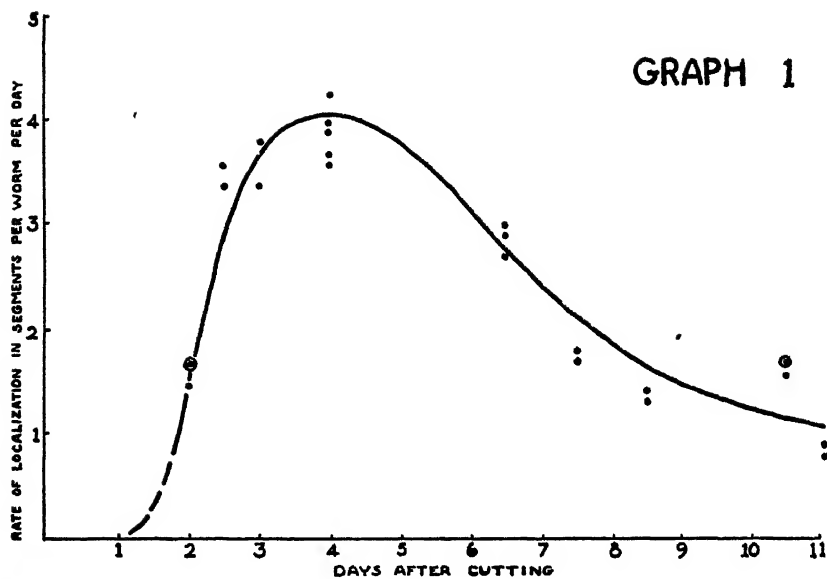
by X-ray treatment showed no similar increase in oxygen consumption. It was considered that the increased oxygen consumption was associated with some process occurring during regeneration. In this preliminary work no counts of segments had been made, but the data of Stone (1932) were used for comparison. It was suggested that differentiation might be the process with which the increased oxygen consumption was associated. It was thought possible that the increase in oxygen consumption might be cyanide-sensitive as in the case of grasshopper embryos (Bodine et al., 1934, 1940). To test this possibility as well as to check results thoroughly using a standard method, experiments were set up in which the  $Q_{O_2}$  of regenerating worms in tapwater and in dilute cyanide solution ( $2 \times 10^{-4}$  M KCN) was determined using Warburg manometers. Detailed observations of the progress of regeneration were made on the same worms. A total of nine groups of worms, 303 individuals, were followed through regeneration in three series of experiments. Four groups of worms, 137 individuals, served as control. Results of the manometric determinations are summarized in Table III, each figure being the average of

TABLE III  
*Oxygen consumption by groups of worms*

Days	Predominating regenerative processes	Regenerating worms		Concurrent control	
		$Q_{O_2}$	$Q_{O_2}^{KCN}$	$Q_{O_2}$	$Q_{O_2}^{KCN}$
2	Mobilization of neoblasts and beginning localization	0.18	0.20	0.17	0.17
3	Localization	0.17	0.16	0.16	0.16
4	Localization	0.16	0.15	0.16	0.15
5	Localization and early differentiation	0.17	0.16	0.16	0.15
6	Early differentiation	0.16	0.15	0.17	0.17
8	Early differentiation	0.18	0.17	0.16	0.15
12	Later differentiation	0.24	0.24	0.16	0.15
13	Later differentiation	0.24	0.25	0.15	0.15

Note: The error when calculating from data on thirty-minute intervals was  $\pm 0.009$ . When calculating from data on the whole run, the error was reduced to about  $\pm 0.003$ . Mean deviation of separate averages from the grand averages listed in this table was  $\pm 0.008$ .

determinations on from two to five groups of worms. Data on progress of regeneration are summarized in Graphs 1, 2, and 3. After two days of regeneration, during which localization occurred in the first new segment from the neoblasts in the blastema, oxygen consumption was slightly above normal. Between three and five days, the period of maximal rate of localization, oxygen consumption was only slightly if at all above normal. While early differentiation was proceeding at its maximum rate six days after removal of the tail, oxygen consumption was strictly normal. But while later differentiation was proceeding, rate of oxygen consumption was increased: at twelve days the rate was 0.08 milliliters per gram per hour higher than the control, i.e., 50 per cent higher. At this time rate of later differentiation was at a maximum. The determinations using the Warburg method confirm in



Note: Mean deviation within each group of worms (twenty-five to forty individuals) ranged from 0.2 to 0.4 segments per worm per day, increasing with time.

general the results of the earlier determinations using individual worms in capillary tubes.<sup>3</sup> At no time was a significant cyanide-sensitive fraction of respiration found.

A summary of data on loss of weight by the worms involved in this study is given in Table IV. These data indicate not only that the regenerating worms lost

TABLE IV

Original length of worms in centimeters	Condition	Average weight per worm at first weighing after removal of tail (2, 3, 4 days)	Average weight per worm at last weighing (12, 13 days)	Average percentage loss per day
3.5 to 4.0	Regenerating	1.36 mg.	0.94 mg.	3.09
	Normal	1.92 mg.	1.57 mg.	1.82
4.0 to 5.0	Regenerating	2.07 mg.	1.57 mg.	3.01
	Normal	2.53 mg.	2.22 mg.	1.51

almost twice as much weight as the control, but also that the initial differences in weight between experimental and control groups due to removal of the tails cannot account for either the increased oxygen consumption or the increased loss of weight. The latter point is clear from a comparison between the larger regenerating worms (4.0 to 5.0 centimeters original length) and the smaller normal worms (3.5 to 4.0 centimeters in length).

### DISCUSSION

Morphogenesis, the origin of form, involves the production of the specific structural chemicals of protoplasm, their orientation into submicroscopic structure, and the summation of submicroscopic structural differences into microscopic and macroscopic structure. Morphogenesis depends upon metabolism in that anabolic processes produce the particular compounds which become oriented into structural parts and catabolic processes must release in usable form such energy as may be required, if any, in morphogenetic processes. Morphogenesis may involve work in the thermodynamic sense according to either of two conditions: (1) there may be free energy of formation of the structure such that the energy becomes resident in chemical and physical structure, and/or (2) the synthesis of materials, their transport to particular positions, and their fixation in these positions, may require energy in such a way that the energy is ultimately dissipated as heat, even as the energy required to move an object horizontally from one point to another is ultimately dissipated as heat.

Tangl (1909), Farkas (1903) and Bohr and Hasselbalch (1903) defined "Entwicklungsarbeit," "work of development," as the total energy dissipated during embryonic development. In a number of different animals the Entwicklungsarbeit was found to be equal to about one-third of the total energy content of the unincubated egg. Needham (1931) criticized the concepts of Tangl and contended that

<sup>3</sup> The differences in general level of oxygen consumption are probably due to the difference in temperature at which determinations were made, i.e., Warburg determinations at 25° C., and capillary tube determinations at 21° C. (Brazda and Rice, 1940, found that oxygen consumption was increased about 40 per cent when the temperature was raised from 25° C. to 30° C.) and to inequality of unit volume to unit weight.

"work of development" should be a potential energy resident in structure, i.e., should be "work of differentiation." He further pointed out in reviewing the work of Bohr and Hasselbalch, that the energy resident in mechanical structure must be either non-existent or extremely small in amount. Essentially, the difficulties arose from the inadequacy of controls: no measure of an energetic cost of maintenance separate from an energetic cost of development was available in the work of Tangl, Farkas, and Bohr and Hasselbalch.

Tyler (1933) found that normally developing dwarf sea urchin embryos consumed more oxygen than normal-sized embryos in reaching the same stages of development. He interpreted his results in terms of a metabolic cost of differentiation. Tyler's concept, different from that discussed by Needham, involves the expenditure of energy in morphogenesis but not necessarily the storage of energy in visible structure. His investigations of giant embryos gave evidence consistent with his interpretation and he tried further to dissociate differentiation, growth, and maintenance in his material, but was unsuccessful. He stated (1936), "It has been concluded from earlier work that energy is required for the processes of embryonic differentiation, although the quantities involved could not be estimated."

Some of the difficulties encountered in studies of morphogenesis in embryonic development are avoided when morphogenesis is investigated in regeneration. Intact animals may be used in measuring the metabolism associated with maintenance,<sup>4</sup> and comparison between the metabolic rates of regenerating and of intact animals gives a measure of metabolism associated with morphogenesis. Increased rate of oxygen consumption during regeneration has been reported in Planarians (Coldwater, 1930). The additional metabolism appeared immediately after cutting and may have been associated with proliferation and increased numbers of the formative cells in this case. Coldwater chose this interpretation. However, it has not been demonstrated that either formative cells or other cells of an embryonic nature have intrinsically high respiratory rate. Tumor cells, for instance, have been found to have a relatively low  $Q_{O_2}$  (Warburg, 1930). Since these may be considered embryonic cells in which determination and differentiation are not proceeding, their metabolism may be considered entirely associated with maintenance and proliferation. On this basis, maintenance of embryonic cells should not, in itself, lead to increased respiration. From an examination of Needham's comprehensive review of the investigations of metabolism of embryos it is clear that the magnitude of rate of oxygen consumption is not greater than that of certain adult tissues. It is possible that in regenerating Planarians the additional metabolism was associated with truly morphogenetic processes rather than with proliferation and maintenance, but the data do not allow a distinction between the possibilities.

In the present work a distinction is possible. During regeneration in *T. tubifex* the rate of oxygen consumption was found to remain normal for the first week. During the second week the oxygen consumption was found to increase to well above normal. Because the markedly increased oxygen consumption did not appear during early stages of regeneration, it certainly was not associated with mobilization or proliferation of the neoblasts. That it was not associated with increase in size

<sup>4</sup> In the present work the data provide an empirical check on this assumption: since the  $Q_{O_2}$  remained normal during most of the first week of regeneration, the aerobic metabolic cost of maintenance of regenerating worms must be the same as that of intact worms.

of the new segments is clear from the consideration that increase in rate of oxygen consumption appeared before marked increase in the size of the new tail; during the first two weeks the regenerant tail constituted only a very small fraction of the total volume of the worm (less than one-twentieth). Hence the additional oxygen consumption may be considered to be associated with morphogenesis proper. From the present data it is not possible to distinguish whether the additional oxygen consumption occurred in the regenerant tail alone or whether the older tissues also consumed oxygen more rapidly. If the increase be referred to the regenerant tail alone, the  $Q_{O_2}$  of this part must have been extremely high. However, in estimating a metabolic cost of morphogenesis it is logical to refer the cost to the worm as a whole. Estimated on the basis of oxygen consumption, the metabolic cost of morphogenesis during the second week of regeneration would be at least half as great as the cost of maintenance of the whole worm. Another estimate of a possible cost of morphogenesis is available from the data. Because of the starving condition of the worms, all substrates of metabolism in both intact and regenerating worms were necessarily derived from the older tissues; loss in weight should be proportional to the amount of the materials metabolized away. Materials which were transferred from the older tissues to the regenerant tissues should not enter into the determination, and thus loss in weight should be proportional to net rather than gross breakdown of substrates. It was found that during the same periods of time regenerating worms lost weight almost twice as rapidly as intact worms. On this second basis, morphogenesis has a metabolic cost nearly the same as the cost of maintenance of the entire worm. The cost estimated from weight loss is thus greater than that estimated from oxygen consumption. Probably not all of the breakdown of substrates is associated with oxygen consumption, and morphogenesis may have an additional cost in terms of activity of anaerobic metabolism or glycolysis.

Analysis of a metabolic cost of morphogenesis may be carried further by comparing the time course of rate of oxygen consumption with the time course of events in regeneration, especially the time course of the processes which were measurable in terms of segments per worm per day. During the period of highest rate of localization, oxygen consumption was only slightly above that of non-regenerating worms. During the earliest stages of differentiation oxygen consumption was almost precisely the same as in non-regenerating worms. This indicates that localization is not particularly costly in terms of aerobic metabolism, while initiation of differentiation appears to cost nothing. The evidence does not indicate that localization and early differentiation are independent of catabolism. Measurements of total oxygen consumption give no indication of the relative activities of different fractions of catabolism, nor do they indicate how much energy may be released through cellular oxidative systems which use some hydrogen acceptor other than molecular oxygen. (Evidence which indicates an "activity metabolism" of localization will be presented in a later paper.)

The peak in rate of oxygen consumption was found to coincide in time with the peak in rate of later differentiation. Furthermore, the earliest time of marked increase in oxygen consumption coincided with the time at which the later stage of differentiation began appearing, first at a low rate, then more rapidly, parallel with increase in rate of oxygen consumption. The marked increase in oxygen consumption in regenerating *T. tubifex* is associated with differentiation, or some process

involved therein, and not with morphogenetic processes in general. It is suggested that differentiation is paid for in terms of metabolically released energy, and that the cost is high. As estimated from increase in oxygen consumption and in loss of weight, this cost must be at least half as great as the cost of maintenance of the entire worm.

#### SUMMARY

1. Methods of measuring "rate of localization," "rate of early differentiation," and "rate of later differentiation," during posterior regeneration in *Tubifex tubifex* have been described.

2. A method of measuring oxygen consumption of individual worms has been described.

3. Oxygen consumption, as determined according to the above mentioned method and also according to the Warburg manometric method, has been found to proceed at a near normal rate during localization and early stages of differentiation in the first week of regeneration.

4. Markedly increased rate of oxygen consumption has been found associated with maximum rate of later differentiation during the second week of regeneration.

5. No significant cyanide-sensitive fraction of respiration was found at any stage of regeneration.

6. Worms in which regeneration had been inhibited by X-ray treatment showed no increase in oxygen consumption.

7. Loss of weight by the starving regenerating worms was found to be almost twice as great as by the intact worms.

8. The data have been discussed in terms of a metabolic cost of differentiation, which cost would be at least half as great as the metabolic cost of maintenance of the entire worm.

9. It is concluded that the marked increase in aerobic metabolism observed during regeneration in *T. tubifex* is associated with some process or processes involved in differentiation.

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# THE EFFECT OF SEX AND AGE ON THE TEMPERATURE AT WHICH REVERSAL IN REACTION TO LIGHT IN ERISTALIS TENAX OCCURS

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## INTRODUCTION

One of the most tantalizing phenomena facing the physiologist is the change of sign of reaction to light that occurs in many organisms. In this reversal an organism which is normally photopositive, moving toward the light, becomes negative and avoids the light, or a normally photonegative organism, moving away from the light, becomes positive. Some even consider this phenomenon "unapproachable," according to Maier and Schnierla (1935). Although this has claimed the attention of extremely able investigators for over 75 years, Holmes' statement (1916) holds good today. "The mechanisms involved are still unknown." The need for information concerning this problem is shown by the brevity of the paragraphs devoted to it by Wigglesworth (1939) and Heilbrunn (1943).

Reversal in reaction to light occurs "spontaneously" with no change in external conditions in *Daphnia* (Ewald, 1914; Clarke, 1932) and in *Spondylomorum* (Mast, 1918). It occurs rhythmically in some animals, according to Bohn (1905, 1907, 1909), Ewald (1914), and Warden, Jenkins, and Warner (1940), and in the course of normal development in others (Fraenkel and Gunn, 1940). For example, lobster larvae (Hadley, 1908) are positive for two days after hatching when they become negative and remain so until shortly before molting when they again become positive. They are negative in the early second stage and third stage but become positive before molting. In the fourth and later stages they are negative.

Various investigators maintain that a reversal of sign of the reaction to light can be produced in many organisms by the following changes: I. In the organism itself: metabolic rate, muscle tonus, water content, concentration of certain hormones, adaptation in sense organs, orientation to gravity, mode of locomotion (e.g., from swimming to crawling), mechanical stimulation, operations (such as removal of all or part of the wings or brain), training, and genetic constitution. II. In the immediate environment: temperature, food supply, background, light intensity (both gradual and rapid), wave length of light, osmotic pressure, oxygen pressure, hydrogen ion concentration, viscosity, and other changes produced by the addition of various inorganic and organic compounds.

Of all these methods none is more important than the use of alterations in temperature to produce changes in the sign of the reaction. An increase in temperature makes some photopositive organisms negative and some, that are negative, positive, while a decrease in temperature makes some positive organisms negative and some, that are negative, positive.

The animals and plants used in previous studies on the effect of temperature on reversal in reaction to light are as follows; swarm spores of *Haematococcus*, *Uloth-*

ria, and other algae (Strasburger, 1878); *Euglena*, *Volvox*, *Spondylomorpha*, and other algae (Mast, 1911, 1918, 1927, 1932, 1936); the flagellate, *Chromulina* (Marsart, 1891); *Rana clamata* (Torelle, 1903); *Arenicola* larvae (Kanda, 1919); *Polychaeta* larvae (Loeb, 1893, 1905, 1906, 1918); *Lumbricus* and *Eisenia* (Prosser, 1934; Mast, 1936); *Cyclops*, *Cypris*, and a water spider (Mast, 1911); certain marine copepods (Loeb, 1893, 1905; Parker, 1901; Rose, 1929); *Daphnia* (Groom and Loeb, 1890; Loeb, 1906; Mast, 1911; Dice, 1914; Rose, 1929; Clarke, 1932); the copepod, *Leptodora* (Siedentop, 1930); *Artemia salina* (Bujor, 1911); *Balanus nauplii* (Groom and Loeb, 1890; Ewald, 1912; Rose, 1929); nauplii of the barnacle, *Chthamalus* (Rose, 1929); various amphipods (Phipps, 1915; Holmes, 1916); *Ranatra* (Holmes, 1905, 1916); *Notonecta* (Essenberg, 1915); the Mayfly nymphs, *Epeorus* and *Leptophlebia* (Allee and Stein, 1918); the beetle, *Anthrenus muscorum* (Janda, 1931); *Drosophila* (Carpenter, 1908); mosquito larvae, *Culex pipiens* (Miller, 1940); the tsetse fly, *Glossina morsitans*, and the stable fly, *Stomoxys calcitrans* (Jack and Williams, 1937).

The small number of species of insects tested furnishes no basis for Holmes' statement (1916), "In the insects reversal of the positive reaction is rather uncommon."

Because nothing is known about the effect of age and sex on the temperature at which reversal occurs in any organism, and because nothing whatever is known about reversal in *Eristalis tenax*, a study of reversal in reaction to light in this insect was made. For this work the drone fly proved as excellently adapted as it has for many other phases of physiological research.

In this paper are presented the results of a study of the effect of sex and age on the temperature at which reversal of reaction to light occurs. *Eristalis* at ordinary temperatures is highly photopositive. Preliminary experiments showed that between approximately 10°C. and 30°C. the flies crawl or fly directly toward a source of light. Outside these limits they are highly negative, moving directly away from a source of light. Since it was found impractical to study in detail the behavior of these flies at low temperatures but comparatively easy at high temperatures, this paper deals almost entirely with the latter.

#### MATERIALS AND METHODS

The apparatus used (Fig. 1) consists of a box made of 6.3 mm. plywood (50 × 35 × 34 cm.). There are two main compartments, a light one, *A*, and a dark one, *B*. The light compartment is lined with white cardboard on the bottom, two sides and one end. The two compartments, 6.25 cm. deep, are separated by a wooden slide, *a*, painted white on the side toward the light compartment, which can be raised. Above the light compartment are two sliding glass panels, *b*, *c*, 6.3 cm. apart. A thermometer, *r* is inserted through a hole, *d* at the level of the white floor, *e*.

The dark compartment, *B*, has the same dimensions as the light compartment, but is lined with dull black cardboard and has a removable wooden cover, *f*, through the center of which a thermometer, *g*, is inserted. A glass window, *h* (3.8 × 12.5 cm.), covered by a removable shade on the outside, is at one end of this compartment.

Below the detachable floors, *i*, *j*, of each compartment the construction is identical. Five centimeters below these floors is a sheet of galvanized steel on which

rests a cardboard, *k*. Below the metal bottoms are two heat chambers, 12.5 cm. deep, *C*, *D*, lined with corrugated cardboard, *l*, and asbestos sheeting, *m*. The heat is supplied by two 100-watt Mazda lamps, *n*, with constant voltage, each lamp being wired separately.

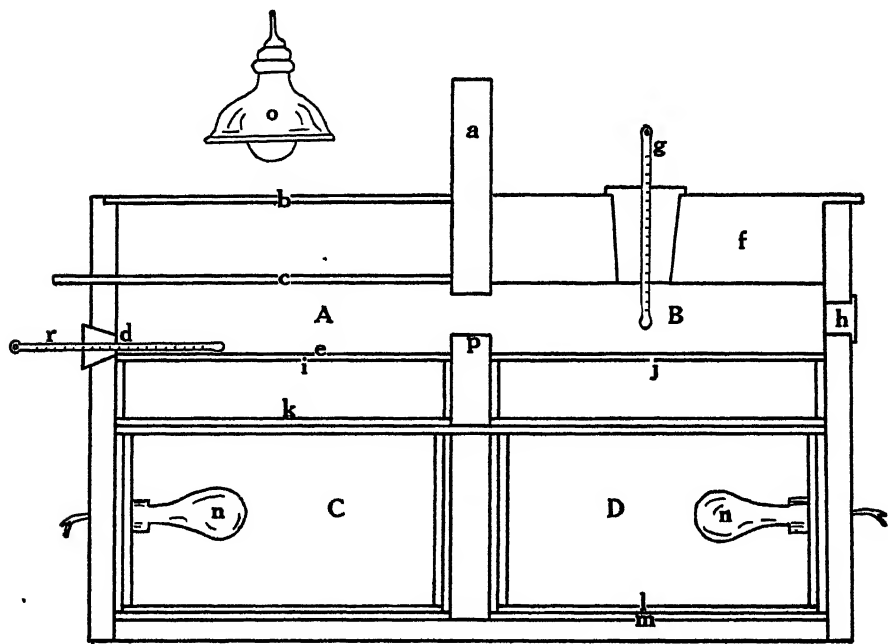


FIGURE 1. Sectional view of the apparatus used. See text.

A 100-watt Mazda lamp, *o*, was suspended 81.25 cm. over the center of the white floor of the light compartment. The luminous intensity on this floor was 700 f.c., as recorded by a Weston exposure meter.

In these experiments only flies of known age and sex were used. They were raised in the laboratory according to the methods previously described (Dolley et al., 1937, p. 410). The exact temperature at which the flies became negative to light and went into the dark chamber was ascertained for six groups of flies whose sex and age are as follows:

1. Young males, 5-16 days.
2. Young females, 5-16 days.
3. Middle-aged males, 28-36 days.
4. Middle-aged females, 28-36 days.
5. Old males, 48-77 days.
6. Old females, 48-77 days.

Two hundred and seventy observations were made on members of each of these groups. No more than three were made on an individual insect and at least twenty-four hours elapsed between successive observations on the same fly.

All experiments were performed in a dark room. A typical experiment was made as follows: The temperature in the dark compartment was raised to between 35° C. and 37° C., that in the light compartment was raised to 28° C. Throughout the experiment the temperature in the dark compartment was seven to nine degrees higher than that in the light compartment. Ten to fifteen flies of the same age and sex, and with unclipped wings, were placed in the light compartment by sliding back the glass plates, *b*, *c*. These plates were replaced; the heating unit under the light chamber was turned on; and the center slide, *a*, was raised, making an opening (18 × 4 cm.) connecting the two chambers, *A*, *B*.

As the temperature in the light chamber rose the flies became restless, crawling and flying about. Soon individuals moved out of the light into the darkness of the dark chamber. A fly was considered to have reversed when it had passed completely beyond the center ridge, *p*. When this occurred the investigator recorded the temperature in the light compartment. The flies followed one another, and one by one entered the dark compartment. Frequently a fly returned to the light compartment after a few minutes in the dark, and then after a few seconds returned again to the dark compartment. Sometimes a given fly made three or four such successive reversals. The temperature at which the final reversal of a given fly took place was recorded and considered one observation. After approximately twenty-five minutes all the flies had reversed and entered the dark compartment. The organisms were then removed and those that had survived were placed in a cage with food and water. They were used for a maximum of two other experiments similar to that described above. An interval of at least twenty-four hours elapsed between successive tests on the same flies. About twenty-five per cent of the insects used in a given experiment did not survive their exposure to the high temperature of the dark compartment.

### RESULTS

The results obtained are given in Figure 2 and Table I. As is shown in this table, the mean temperatures in degrees centigrade at which the flies reversed are as follows: old males,  $33.18 \pm 0.09$ ; old females,  $33.822 \pm 0.091$ ; middle-aged males,  $34.450 \pm 0.088$ ; middle-aged females,  $35.06 \pm 0.11$ ; young males,  $35.500 \pm 0.099$ ; young females,  $36.106 \pm 0.082$ . The standard deviations (Table I) are as follows: old males,  $1.485 \pm 0.064$ ; old females,  $1.489 \pm 0.064$ ; middle-aged males,  $1.440 \pm 0.062$ ; middle-aged females,  $1.742 \pm 0.075$ ; young males,  $1.623 \pm 0.070$ ; young females,  $1.318 \pm 0.057$ .

Are the differences between the mean temperatures just given at which the two sexes reversed significant? According to Pearl (1940, p. 287), "the odds are 369.4 to 1 against the occurrence of a deviation in either the plus or minus direction as great or greater than  $3 \times \text{S.E.}$  These are long odds, and are conventionally regarded as amounting to practical certainty."

The differences between the means of the two sexes of the old, middle-aged and young flies are, respectively, 5 +, 4 +, and 4 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively, over 1,744,000; 15,770; and 15,770 to 1. It is evident that the differences between the means of the sexes in the above three age groups are clearly significant. Consequently, it is obvious that in each group the female flies reversed at a higher temperature than the male flies.

Age also has a definite effect on the temperature of reversal. This is shown when the observations on the individuals, both males and females, of the same age, are put together, as is done in Figure 2 and the lower portion of Table I. It is clear from this figure and table that the mean temperatures at which the old, middle-aged and young flies reversed are:  $33.50 \pm 0.07$ ,  $34.781 \pm 0.071$ , and  $35.786 \pm 0.064$ , respectively. The differences between the means of the old and middle-aged, the old and young, and the middle-aged and young flies are, respectively, 12 +, 22 +, and 10 + times the standard errors of the differences. Consequently, age is an important factor in determining the temperature at which *Eristalis* changes its reaction to light. The younger the fly, the higher the temperature at which reversal occurs.

TABLE I

*The effect of sex and age on the temperature at which reversal in reaction to light in Eristalis occurs. See text*

Age	Sex	Mean temperature in degrees centigrade $\pm$ standard error	Standard deviation $\pm$ standard error
Old	male	$33.18 \pm 0.09$	$1.485 \pm 0.064$
	female	$33.822 \pm 0.091$	$1.489 \pm 0.064$
Middle-aged	male	$34.450 \pm 0.088$	$1.440 \pm 0.062$
	female	$35.06 \pm 0.11$	$1.742 \pm 0.075$
Young	male	$35.500 \pm 0.099$	$1.623 \pm 0.070$
	female	$36.106 \pm 0.082$	$1.318 \pm 0.057$
Old		$33.50 \pm 0.07$	$1.522 \pm 0.046$
Middle-aged		$34.781 \pm 0.071$	$1.652 \pm 0.050$
Young		$35.786 \pm 0.064$	$1.477 \pm 0.045$

It is patent that in the young flies the females with a standard deviation of  $1.318 \pm 0.057$  showed less variation than did the males with a standard deviation of  $1.623 \pm 0.070$ ; that in the middle-aged series the females showed greater variation than the males; that in the old flies the variation in the two sexes was about the same; and that between the three groups of different ages, each being composed of flies of both sexes, the young flies showed the least variation. The significance of the differences given above is not at present known, but it is true that the young flies were a more homogeneous group so far as age is concerned than were the old flies. The maximum difference in age between the members of the young series was eleven days, while the maximum difference in age between the members of the old series was twenty-eight days, a long period of time in comparison with the duration of life of *Eristalis*.

The reversals described above are unquestionably reversals to light and not to heat energy. The flies in going from the light compartment into the dark compart-

ment were not going from a region of high temperature to one of lower heat energy. The reading on the thermometer (Fig. 1, *r*) in the light compartment was compared with that of a black bulb thermometer made by coating a thermometer with lamp black. During the experiments the temperature as recorded by the latter was only 1.5 degrees higher than that recorded by the one used. Consequently, even if the flies had absorbed as much heat energy as did the black bulb thermometer, still the effective temperature in the dark compartment would have been from 5.5 to 7.5 degrees higher than that in the light compartment.

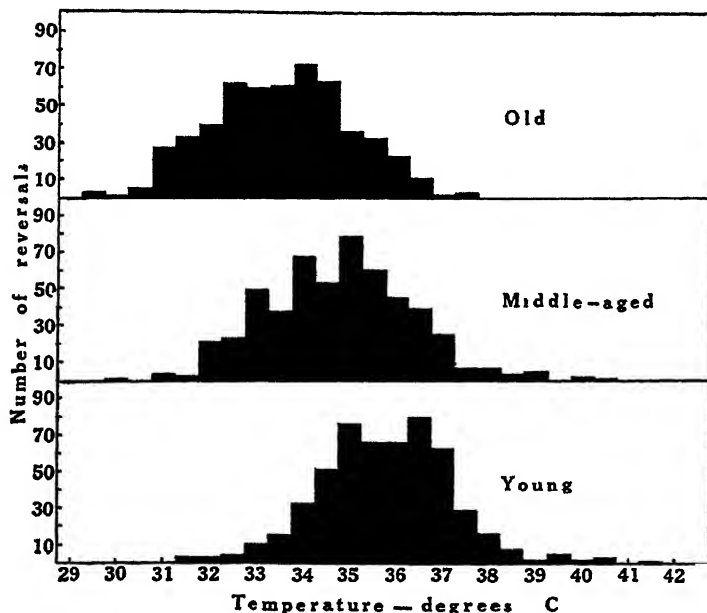


FIGURE 2. Histogram showing the effect of age upon the temperature at which reversal in reaction to light in *Eristalis* occurs. Note that the means of the reversals for the older flies are at lower temperatures than are the means for the younger ones. See Table I.

### DISCUSSION

The results presented in this paper show that the temperature at which *Eristalis* reverses in its reaction to light is not correlated specifically with either a decrease or an increase in heat energy for it can be caused by both. Reversal in sign of reaction occurs both when the temperature is raised above a certain point and when it is lowered below approximately 10°C., as stated previously.

This conclusion is in harmony with the following: 1. Mast's contentions (1918) that a decrease in heat energy and an increase in light energy produce similar effects in photopositive euglenae and other algae, and (1936) that reversal in *Volvox* and *Euglena* is due to "internal changes" and "is not specifically correlated with the immediate environment"; 2. the conclusion of Phipps (1915) that reversal in certain amphipods is associated with changes in their "physiological states"; 3. that of Welsh (1930) that reversal in the water mite, *Unionicola*, "is probably a central nervous phenomenon"; 4. that of Washburn (1936, p. 208) that the "complex influ-

ence of external and internal conditions on phototropism" . . . "is based on innate factors"; 5. that of Allee and Stein (1918) that reversal in reaction to light in certain Mayfly nymphs is associated with either increase or decrease in metabolic rate; and 6. those of Jack and Williams (1937) that if the temperature is raised sufficiently high the normally photopositive reactions of the tsetse fly, *Glossina morsitans*, and the stable fly, *Stomoxys calcitrans*, become reversed, and that "the temperature at which the negative reaction develops appears to depend somewhat upon the flies' physiological conditions."

The conclusion stated above is not in harmony with the following: 1. Davenport's contention (1908, p. 200) that a "Diminution of temperature below the normal causes reversal of the normal response, elevation of the temperature to near the maximum accelerates the normal response"; 2. Maier and Schnierla's conclusion (1935) that the sign of the reaction to light in an organism "depends upon the characteristic metabolic condition of its species"; and 3. that of Holmes (1905) for *Ranatra* that any condition causing an increase in activity accentuates positive reactions and any quieting conditions make them negative.

The results presented in this paper show also that the temperature at which reversal occurs in *Eristalis* depends upon the resistance of the organism to the injurious effects of temperatures above and below the normal. The nature of this resistance is as yet unknown. Old flies are less able to endure the effects of abnormal temperatures than are young ones. Since the temperature at which reversal occurs in a given intensity of light is lower in old than in young flies, and in males than in females, it is probable that females are more resistant to the effects of temperatures outside the normal range than are males. This conclusion is confirmed by the results of work now in progress.

Reversal in response to light in *Eristalis* produced by changes in temperature is probably due to a different mechanism from that involved in the reversals occurring in normal development, which are doubtless associated with the development or degeneration of photosensory or other organs. As stated previously (Dolley and Haines, 1930), *Eristalis* larvae are highly positive to light for the first few hours after hatching. They then become negative to light and remain so until they pupate. The imagoes are at first negative but soon become positive at ordinary temperatures, and remain so throughout their lives. Similar phenomena have been described for blowflies (Hermis, 1911; Gross, 1913; Patten, 1916), *Amaroucium* larvae (Grave, 1920; Mast, 1921), and for other organisms. According to Pause (1918) Chironomus larvae are positive until they have formed haemoglobin when they become negative.

#### SUMMARY

1. Over 1,620 observations were made on over 1,000 flies in ascertaining the temperature at which *Eristalis tenax* becomes negative to light.

2. In a luminous intensity of 700-foot candles *Eristalis* is highly photopositive within a temperature range between approximately 10° and 30°C. Outside these limits it is highly negative. Reversal of the photopositive reaction can be produced either by increase or decrease of the temperature.

3. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends on the sex of the flies. Females cease their positive reaction to light and become negative at a higher temperature than do males.

4. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends also on the age of the flies. The younger the fly, the higher the temperature at which it ceases its positive reaction to light and becomes negative.

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## OBSERVATIONS ON THE REPRODUCTION OF THE SPINY DOGFISH, *SQUALUS ACANTHIAS* \*

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The elasmobranch fishes offer an exceptional opportunity for studies of the physiology of reproduction. Within this old and diversified group of vertebrates many species are oviparous, others are ovoviviparous, and some are viviparous, thus indicating various degrees of physiological dependence of the young on the parent. Ranzi and his collaborators have made comparative studies of several representative species and their results and the observations of other workers have been presented in several reviews (see Needham, 1942, for discussion and references).

Our observations were made on two species of dogfish, *Squalus acanthias* (spiny dogfish) and *Mustelus canis* (smooth dogfish), common in the vicinity of Woods Hole, Massachusetts. The spiny dogfish is ovoviviparous and maintains its young free in the uterus while the smooth dogfish is viviparous and has young whose yolk sacs are modified into placental structures that are firmly attached to the uterine endometrium during the last two-thirds of pregnancy. Our chief interest was a study of the endocrines that might be associated with gestation in these two species of dogfish. This approach was quite different from the investigations of Ranzi and others which were primarily concerned with foetal nutrition. However, it was found that *Squalus acanthias* was not suitable for our experimental purposes. They could not withstand necessary surgical procedures nor could they live for long periods though kept in large, outdoor live-cars. Consequently, the results of our studies on *Squalus acanthias* were mostly observational. These are reported here while the results of our experiments on *Mustelus canis* will be published separately.

### GENERAL OBSERVATIONS ON THE REPRODUCTIVE CYCLE

The different times at which *S. acanthias* is present at various points along the Atlantic coast indicate that it migrates northward in the spring and southward in the fall. It appears in Buzzards Bay and Vineyard Sound in late April or early May. It becomes very abundant during May but declines in number by the end of the month and is caught only infrequently toward the close of June. Correlated with its disappearance from the vicinity of Woods Hole is its arrival at successive points farther north, reaching the coastal waters of Newfoundland the last of June or early July. The migration southward apparently begins in September and spiny dogfish are again caught in large quantities by fishermen at Woods Hole in October and November as they pass by Cape Cod.

Our studies are based on animals obtained during the spring and fall migration, a period of about six months. Consequently we can only infer what changes might

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have occurred in the reproductive system during the winter and very early spring. However, we have examined several hundred spiny dogfish since our investigations were started in 1936 and believe that we have a fairly accurate account of the reproductive cycle and duration of pregnancy. Some of our observations are corroborations of certain details of the reproductive cycle well known to fishermen and collectors at Woods Hole, while others confirm the results of similar studies on *Squalus acanthias* by Hickling (1930) and Templeman (1944) in Newfoundland, and by Ford (1921), Ranzi (1934) and Popovici (1938) on *Acanthias vulgaris* as found in the English Channel, Mediterranean Sea, and Black Sea.

Most of this work was done at the Oceanographic Institution in Woods Hole and we wish to express our sincere thanks for generous laboratory accommodations. We also are indebted to Dr. H. B. Bigelow and Dr. W. C. Schroeder for many helpful suggestions and to Mr. James McInnis of the Marine Biological Laboratory, Supply Department, who made it possible for us to examine many hundreds of dogfish.

#### UTERINE YOUNG

The sexually mature females, when they arrive at Woods Hole during May, can be divided into two distinct groups depending on the stage of development of their embryos. For convenience we can designate these two periods of gestation as Stage *A* and Stage *C* (Fig. 1). Those females belonging to Stage *A* have ovulated recently while those of Stage *C* have fetuses 12 to 20 cm. in length. Females that are 80 cm. to a meter or more in length are pregnant almost without exception and all are invariably in either Stage *A* or Stage *C* of gestation. The pregnant females are not always equally divided between these two stages. In 1937 they were about equal but in 1938 there were about four in Stage *A* to one in Stage *C*.

The uterine ova of animals in Stage *A* are enclosed in a membranous envelope forming a structure commonly called a "candle." There is usually a candle in both the right and left horn of the uterus and each contains one to four ova. The average wet weight of 26 candles containing one to 4 ova (total of 65 ova) was 46.2 grams per ovum. The development of the embryos is about the same, each ovum having a blastoderm somewhat comparable to that of a hen's ovum at about 16 to 20 hours incubation.

The pups of Stage *C* have large pendent yolk sacs and are free in the uterine lumen. The range of variation in length is considerable (12 cm. to 20 cm.) and also there is a great difference in the size of the yolk sac, even among pups of the same length. Consequently there is a wide variation in the combined weights of the pups plus their yolk sacs ranging between 30 to 60 grams wet weight.

When the dogfish return to the vicinity of Cape Cod in October and November, the adult females again can be divided into two groups depending upon the condition of pregnancy. These two groups have been designated as Stage *B* and Stage *D* (Fig. 1). The ova of Stage *B*, though much further developed than those of Stage *A*, are yet enclosed in the candle membrane. The embryos are from 3.5 to 7.5 cm. long and are connected by an umbilical stalk about one inch in length to a large yolk sac about the size of the original ovum. The average wet weight of the embryo plus the yolk sac is about 40 grams.

Some of the females of the other group, Stage *D*, have given birth and consequently their uteri are empty while those that have not given birth have large pups

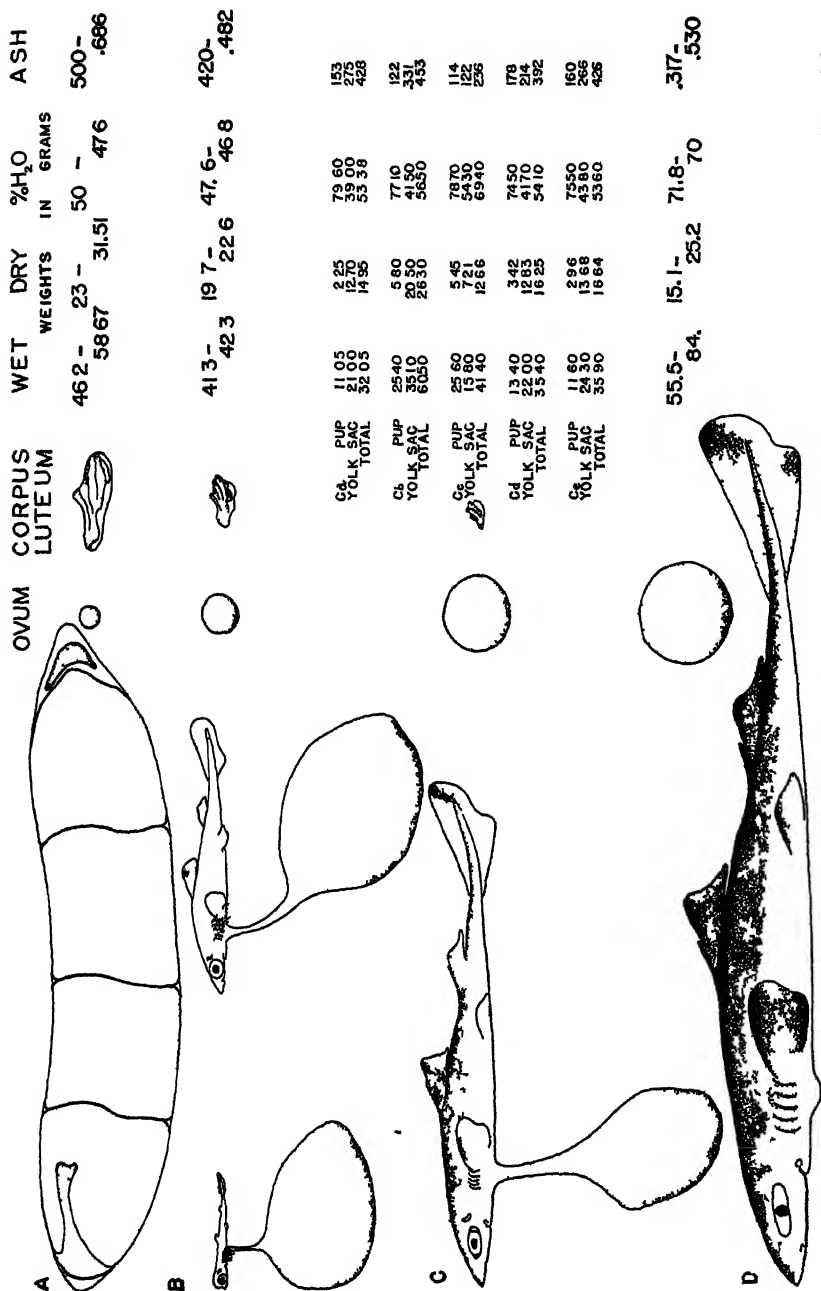


FIGURE 1. Comparison of the four stages of gestation in the spiny dogfish *Squalus acanthias* as seen at Woods Hole Massachusetts, in late April and May (Stages A and B) and October and November (Stages C and D). *O. m. luteum* Progressive increase in size of ovarian ova with the advancement of pregnancy. *C. m. luteum* Gross appearance of the involuting corpora lutea. Drawings 1/4 natural size.

that are 23 to 29 cm. in length. The yolk sac of some of these pups has been withdrawn into the body and the umbilicus is yet open while in others it is yet present though greatly reduced. The wet weight of such pups varies between 55 and 85 grams.

These four groups of uterine young obviously represent four different stages of gestation. When arranged in order of degree of development, which also agrees with the sequence of time, it readily is seen that the period of gestation is surprisingly long.

#### OVARIAN OVA

When the ovaries of pregnant spiny dogfish representing the four stages of gestation are compared, they too show four corresponding stages in the development of the ovarian ova (Fig. 1). The early development of the embryos of animals of Stage *A* indicates that ovulation has occurred quite recently and consequently large ovarian ova are absent. Instead, there are numerous, usually 50 or more, small marble-white follicles the size of peas that contain ova usually weighing less than one gram. The embryos of Stage *B* are more advanced and likewise the ovarian ova. The largest eggs in the ovary are about the size of Concord grapes and have begun to take on a yellowish color due to the storage of yellow yolk.

The ovaries of Stage *C* females contain developing ova each of which usually weighs about 9 grams wet weight (variation 4 to 15 grams, average for 264 eggs, 9.1 grams). There are usually two or three such ova in each ovary, the total number never exceeding eight or nine, and they are not necessarily divided equally between the right and left side. They represent the next generation of ova that will be ovulated after the termination of the existing pregnancy and their average number is in close agreement with the average number of young found at one time in the uterus.

The average number of large ovarian ova for Stage *D* is about the same as that for Stage *C* but the eggs are considerably larger, being about 3 cm. in diameter and weighing between 30 and 40 grams. These weights are from two-thirds to three-fourths that of the candle ova of Stage *A* and strongly suggest that the eggs of the spiny dogfish at the time of ovulation probably average close to 50 grams. The fish of Stage *D* are seen at Woods Hole in October and November and the difference between the weight of the ovarian ova at that time and the ova in the candles of Stage *A* should give a fairly accurate estimation of the weight of the egg at ovulation, which probably occurs in February or March.

Thus it is seen that the ovaries of the four different groups show a progressive development of ovarian ova. Those of Stage *A* are small and numerous while in the succeeding three stages of gestation there is an increase in size and a decrease in number. It is, of course, quite common among the vertebrates for many small Graafian follicles to start developing even though only a very few ova are ovulated. In the dogfish as elsewhere the decrease in number of follicles is brought about by atresia. Most of such elimination must occur before Stage *C*, as we did not see large follicles that were undergoing atresia in either Stage *C* or *D*. Nor did we find large follicles in Stage *A* that had failed to ovulate and consequently were being resorbed.

## CORPORA LUTEA

One of the interesting features associated with gestation in the dogfish is the formation of corpora lutea in the ruptured follicles following ovulation. Aside from the question of their physiological importance, the corpora lutea are useful in determining the time sequence of pregnancy as they undergo definite morphological changes as gestation proceeds.

The number of corpora lutea in the right and left ovary varies considerably. There may be six corpora in one ovary and none in the other or their distribution may be 4 and 4, 2 and 6, 3 and 3, 1 and 4, 2 and 4, etc. There is almost always an exact correlation between the number of corpora lutea and the number of embryos in the uterus. However, there is no correlation whatever between the number of corpora in any ovary and the number of embryos in the corresponding uterine horn on that side. The number of embryos in the candles of the right and left horns of the uterus tend to be evenly divided regardless of the unequal distribution of the corpora lutea between the ovaries. It seems that at ovulation an ovum may enter either horn of the uterus in a way probably similar to that described by Metten (1939) for *Scylliorhynchus canicula*. However, in *S. acanthias* Templeman (1944) found, from a statistical study of several hundred animals that the number of young tends to be greater in the right uterus than in the left.

The corpora lutea of Stage A (Figs. 1, 2, 3) are large wrinkled sac-like structures 2 cm. or more across at the greatest diameter. Each is surrounded by a broad tissue space containing a clear fluid and consequently can be separated easily from the ovarian stroma and removed. Histological examination of such corpora shows a remarkable modification of the follicular wall which is thrown up in tall folds and drawn out in long lacy extensions into the follicular cavity. The luteal tissue seems to be derived entirely from the follicular granulosa.

The cells of the young corpus luteum first become very tall and have large vacuoles and irregular external cell borders (Fig. 2). They apparently have the ability to take up solid particles from the cavity of the ruptured follicle as occasionally a yolk granule is found in such cells. The ingestion of yolk by luteal cells is a common observation in the smooth dogfish and especially is this so during atresia of large follicles. Such activity of the corpus luteum will be described more fully in a subsequent report on the smooth dogfish.

The cells of the theca interna that are taken into the folds of the developing corpus do not take on a glandular appearance but contribute to the thin connective tissue framework that supports a rather generous supply of blood vessels. Cells derived from the granulosa are sharply separated from the underlying connective tissue structures.

There is considerable variation in the appearance of the granulosa-luteal cells of the corpora lutea of Stage A which seems to be correlated with age. The first reactions of the granulosa following rupture of the follicle and ovulation result in the development of the structure just described (Fig. 2). Other corpora are found, in animals of this stage, that seem to be older (Fig. 3). These are somewhat smaller and their walls thicker. The luteal cells have lost considerable vacuolation and are more tightly pressed into a folded epithelial-like lining of the luteal cavity.

Females with candle embryos taken in October and November, Stage B, have corpora lutea that are definitely older than those found in Stage A. This difference

in age is shown by a decrease in size of the luteal body and a reduction in size of the luteal cells which now have irregular shaped nuclei (Fig. 4). Also, those cells that lie next to the luteal cavity have become loosened and scattered through the luminal fluid, while the connective tissue of the folds has been modified into very conspicuous membranous partitions.

The corpora lutea of Stage *C* are smaller than those of Stage *B* and the luteal tissue is undergoing degeneration as shown by vacuolation and disintegration of the cells (Fig. 5). These changes must progress very slowly as the luteal bodies



FIGURE 2. Corpus luteum of Stage *A* showing changes that take place in the granulosa soon after ovulation.  $\times 100$ .

can yet be distinguished in Stage *C* where they have been reduced to small yellow structures only 3 or 4 mm. in diameter. The luteal cells of these structures have lost most of their cytoplasm and about all that remains are small cells with picnotic nuclei scattered through the shrunken connective tissue framework of the original corpus luteum.

In Stage *D* very small, compact, yellow masses of tissue can be found that seem to be all that remains of the luteal bodies. Histologically, they are composed of small cells of irregular shapes with little cytoplasm and dense nuclei loosely distributed in the connective tissue skeleton of the old corpus luteum (Fig. 6).

These observations indicate that the corpora lutea in the spiny dogfish remain in the ovary for the better part of two years and during this time they undergo a progressive decrease in size and histological degeneration.

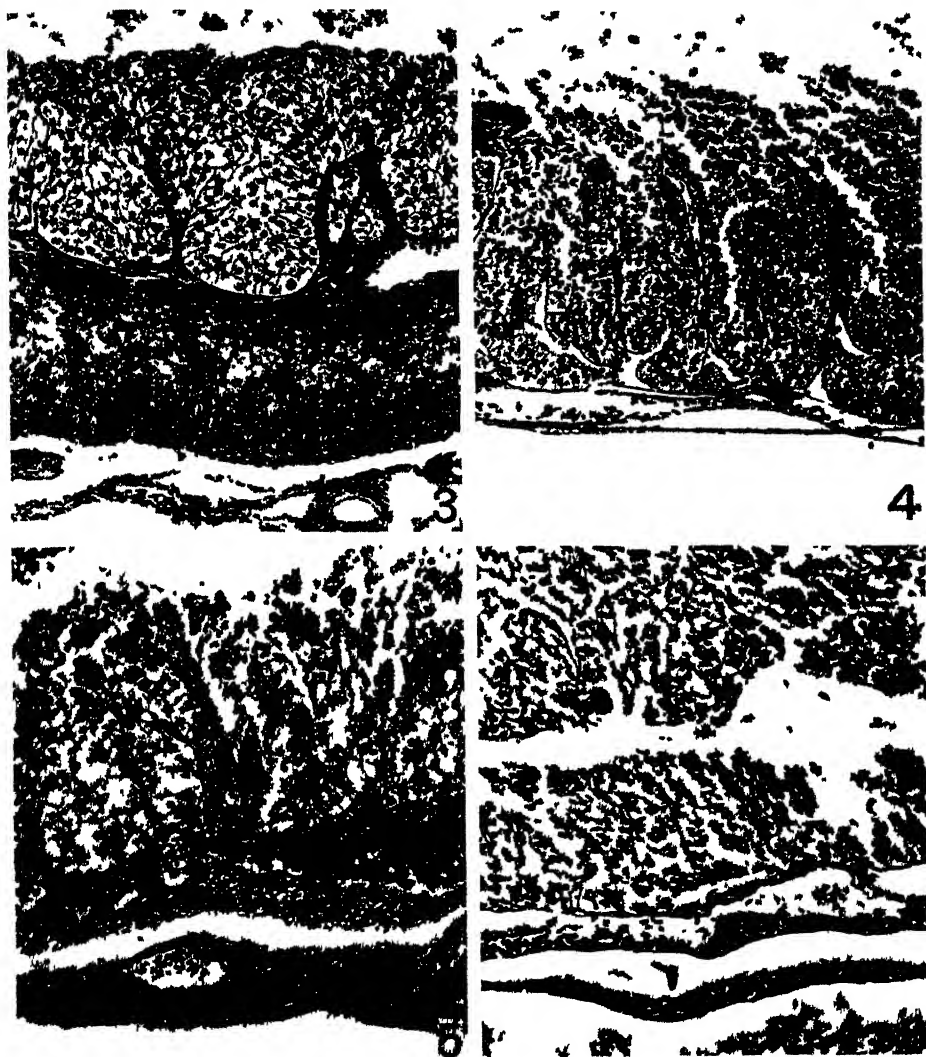


FIGURE 3. Corpus luteum of Stage *A* somewhat older than that shown in Figure 2. The granulosa luteal cells have lost their vacuoles, decreased in size and become organized into a folded multi-layered lining of the follicular cavity.  $\times 100$ .

FIGURE 4. Corpus luteum of Stage *B*. The corpus luteum has decreased in size by at least one-half, and consequently the folds are more compressed. The luteal tissue is degenerating as indicated by a general decrease in size of the cells, small irregularly shaped nuclei and fraying with release of cells into the lumen.

The connective tissue inclusions between the folded granulosa luteal cells of the corpus luteum can be seen distinctly.  $\times 100$ .

FIGURE 5. Corpus luteum of Stage *C*. Such corpora are about one year old and show advanced degenerative changes.  $\times 100$ .

FIGURE 6. Corpus luteum of Stage *D*. Corpora of this stage are 18 to 20 months old and are reduced to small, yellow, compact bodies 2 to 3 mm. in diameter.  $\times 100$ .



## NUTRITION OF THE EMBRYO

The embryos of *Squalus acanthias* develop free in the uterine lumen and possess no obviously specialized structures for obtaining nutritive materials from the uterine endometrium. Judging from their morphology they meet the requirements of a typical case of ovoviviparity. This and their extraordinarily long period of gestation of almost two years aroused interest in the problem of dependence or lack of dependence of the embryo on the parent as a source of nutrition. Consequently, representative material was collected from animals in the four stages of pregnancy we have described (Fig. 1) and data obtained on wet weight, dry weight, percentage of water and ash content. We have already mentioned the rather wide variations in size and weight of both ova and embryos so it is quite possible that our data are not sufficient in range and numbers to be considered as exact for the four stages of development represented, but we believe they show at least the general situation.

The ova of Stage *A* are surrounded by the thin membrane of the candle and, as they usually burst when this envelop is opened, it is extremely difficult to obtain weights of individual ova. Consequently, the whole candle was weighed, the small amount of fluid beneath the membrane at each end was withdrawn as completely as possible and the ova released into a dish by rupturing the envelop. After deducting the weight of the fluid and membrane the average weight of the ova was determined. Twenty-six candles, each containing one to four ova, when treated in this way gave an average weight of 46.2 grams per ovum. Seven ova, representing the upper limit of variation in weight, taken from two candles (3 in one and 4 in the other) of a large fish averaged 58.67 grams. This material was used for making the analyses shown in Figure 1.

In the study of Stage *B* an attempt was made to select embryos from the two extremes in the variation of development. At first, separate determinations were made for the embryo and yolk sac but for the smaller embryos the water content was so high and the amount of solids so small that it was thought a combined analysis would be better for the rather general purpose in mind. Such embryos are about 3.5 cm. long, weight 0.259 gm., and are 90 per cent water.

The pups of Stage *C* vary between 12 and 20 cm. in length, and they have yolk sacs of widely different sizes. From the standpoint of selecting material for analyses, this is complicated further by the fact that pups of the same length may have yolk sacs so different that one may be three times the weight of the other. Consequently, young that seemed to be in general representative samples of the upper and lower range in length were used for making the determinations shown in Figure 1.

Three small pups 12.5, 13, and 13.5 cm. long having an average wet weight of 11.05 gm. and dry weight of 2.25 gm. had an average total ash of 0.153 gm. Their yolk sacs averaged 21.0 gm. wet weight, 12.7 gm. dry weight and had an average ash content of 0.275 gm. The average combined ash of yolk sac and pup was 0.428 gm. A large pup 20.2 cm. long weighing 25.4 gm. wet weight, 5.8 gm. dry weight had an ash content of 0.122 gm. Its yolk sac weighed 35.1 gm. wet weight, 20.5 dry weight and had 0.331 gm. of ash. The combined weight of ash for the pup and yolk sac was 0.453 gm. A pup of about the same length (19 cm.) and weight (25.6 gm. wet weight, 5.45 gm. dry weight) but with a yolk sac only about half as large (15.8 gm. wet weight, 7.21 dry weight) had a combined ash content of 0.236 gm. (pup 0.114 gm., yolk sac 0.122 gm.).

These results have several points of interest. First the total ash of the young of this stage is not much different from that of Stage *B* although it is divided between the yolk sac and pup. Also the amount of ash is less than that found for the ova of Stage *A*. Another result that seems remarkable and is not understood is the relatively high ash content of the small pups.

The material used for the study of Stage *D* consisted of two large pups. The largest was 29 cm. long, weighed 84 gm. wet weight, 25.2 gm. dry weight, and had a total ash of 0.530 gm. The yolk sac was completely absorbed but the umbilicus was yet open (Fig. 1). The other was 24.5 cm. in length, weighed 55.5 gm. wet weight, 15.1 gm. dry weight and had a total ash of 0.3169 gm. This fish had a small yolk sac about 2 cm. in diameter (Fig. 1).

It seems obvious that the large pup was about ready to be born and equally obvious that the smaller pup never could have attained the size of the larger before parturition. Therefore there must be a wide difference in both length and weight of the young at birth. However, similar variations were found in all of the four stages of gestation that were studied, and these observations are in agreement with those made by Templeman. It seems reasonable to suspect that the size of a pup at birth may be correlated with the size of the ovum from which it came. If we make this assumption, then in our analyses, the large pups should be compared with the large ova and the small pups with the small ova. If the results for total ash are compared on this basis it is at once clear that the ash of the ovum exceeds that of the pup.

Therefore, it appears that the developing young of the spiny dogfish need not depend on the parent for inorganic matter. However, the presence of more inorganic matter in the egg than in the young at term does not preclude the possibility of a differential loss and absorption of mineral salts. Determination of the components of the ash would be helpful in answering this question but probably would not be conclusive.

Our data also indicate a loss of organic matter during development and an increase in water. When the ova of Stage *A* are compared with the mature pups of Stage *D* it is seen that the dry weight of the small ovum is 8.1 grams heavier than that of the small pup and the difference between the dry weights of the large ovum and large pup is 6.31 grams. The small pups also show a 75 per cent gain in water and the large pups a gain of 85.8 per cent.

These data were obtained from a few selected samples that we thought were representative of the four stages of gestation as seen at Woods Hole, Massachusetts. Therefore they should not be considered as being quantitatively exact but rather as showing the general trend of events taking place during development. It is definitely certain that the developing young obtain water from the parent, but whether they are entirely independent with regard to organic and inorganic nutrients is yet a question. However, these observations indicate that gestation in *Squalus acanthias* goes on under ovoviviparous conditions.

## DISCUSSION

Thus it is seen that the mature females caught at Woods Hole in the spring and fall are carrying young, almost without exception, and that they can be divided into four distinct groups on the basis of foetal development, size of ovarian ova and condi-

tion of the corpora lutea. These groups are so characteristic that no overlapping was found in the examination of several hundred fish. In fact it is extremely simple to determine the group to which a fish belongs by the gross appearance of the ovaries and uterine young.

It seems unmistakable that these four groups represent four stages of a gestation that extend over the better part of two years, probably 20 to 22 months. It is true that at present we can only surmise what takes place during the six months from November until May while the fish are on their southern migration. However, our records are fairly complete for the period between late April and November while these fish are in the waters along the New England coast.

When the fish arrive at Woods Hole in late April and May the embryos of the candle ova are small blastoderms and, judging by the slow rate of development, ovulation probably occurs in February or March. It is assumed that these fish (Stage A) as seen in April and May, are the same as those having candles in November (Stage B). Therefore, the difference between Stage A and Stage B represents the development that occurs in about the first six months of gestation, most of which is spent between Cape Cod and Newfoundland.

We also think that the fish in Stage B are those which, after their southern migration during the winter, return to Cape Cod the following May as fish in Stage C. During this period of about six months the uterine young increases in length from embryos of 3.5 to 7.5 cm. to pups of 12 to 20 cm.

These fish of Stage C, when next seen at Woods Hole in October and November, are in Stage D of gestation and a few have given birth to their young. The uterine young vary in length from about 24 to 29 cm. In some the yolk sacs are entirely withdrawn into the body while in others they are yet present but reduced to about 2 cm. in diameter. The indications are that the majority of births occur in late fall somewhere south of Woods Hole and that the pups are about 25 to 30 cm. in length when born.

Thus it seems that the only plausible explanation for these observations is that the spiny dogfish gives birth to young every other summer she visits the New England coast, and ovulates every other spring shortly before or soon after she starts on her northern migration. Furthermore, the female population is composed of two groups whose reproductive cycles are separated by about one year, and the four stages of gestation represent periods of approximately six months. Thus, each spring at Woods Hole the population includes only Stages A and C and in the fall only Stages B and D.

Our knowledge of the seasonal migration of *S. acanthias*, though fragmentary, tends to support the points just made concerning the relationships of the development of uterine young. Where the spiny dogfish spends the winter is a question that has not been answered but it is probably in deep water off the coast in the latitude of North Carolina. From there, judging from the observations of Bigelow and Welsh (1924), they arrive each spring in April almost simultaneously along the coast from Cape Lookout to Long Island. They usually appear at Woods Hole the last week of April or the first of May and are also found at about this season on Georges Bank. There seems to be a general migration northward from these points. Spiny dogfish do not appear in Massachusetts Bay before May, and Templeman (1944) reports their arrival along the coast of southern Newfoundland by

the middle of June and northern Newfoundland and Labrador by the second week of July.

Newfoundland and the southeastern coast of Labrador is the northern limit of migration. An additional point of interest is that by the time they become abundant in these waters they have almost entirely disappeared from around Cape Cod and points farther south. However, in Newfoundland, they arrive in June, become abundant in July and remain so until September or October. In contrast with this, they visit Woods Hole twice a year, in the spring and fall. Therefore, during their summer migration, which covers a period of about six months, approximately half of the time is spent in the general vicinity of Newfoundland. Judging from the observations made by Templeman the adult females are the first to arrive in the spring and apparently are the first to start south in the fall. In late November most of those caught were mature males, and immature males and females while only a few mature females were taken.

If the spiny dogfish make this round trip annually from their winter quarters, it is obvious that they must be capable of swimming great distances in a surprisingly short time. That they can do this is indicated also by Templeman's studies. He tagged 279 dogfish, mostly adult females, in the vicinity of St. John's Newfoundland between July 9th and 23rd and several were caught elsewhere in the same year. One of these, an adult female, was taken 132 days later close to Thatcher's Island Buoy, about 1,000 miles from the point where it was tagged. To travel such a distance this fish would have to average something like 7.6 miles per day even if it swam in a straight line which it is reasonable to believe it did not do. Thus it seems that at least the adult females are capable of covering what we believe is their migratory route.

A rather interesting deduction concerning migration also can be made by comparing the constitution of the population of spiny dogfish in Newfoundland and Woods Hole. Templeman states that the smallest dogfish caught was 58 cm. in length. At Woods Hole, small dogfish 40 to 50 cm. in length make up a considerable part of the general population. This indicates that these small fish, probably yearlings, do not reach the northern limit of the migratory range.

Certain differences in the stages of development of candle embryos and large uterine young of the fish examined by Templeman and ourselves also seem significant for determining migration. However, exact comparisons cannot be made as we did not make a large number of measurements that can be subjected to statistical treatment, but it is important to note extremes in size of candle embryos and large uterine young. The embryos of Stage A taken at Woods Hole in May were yet only blastodermic discs with an occasional exception in which a very small embryo could be seen. Of 139 comparable fish taken in Newfoundland in July, 104 had eggs showing blastodermic discs while the others had small embryos, the largest found in two animals being 1.8 and 2.0 cm. respectively. No embryos comparable to these largest ones have been seen at Woods Hole in May, and the fact that the great majority were yet blastodermic discs in July indicates that the rate of development is slow. However, in August, Templeman found only one fish in a group of 42 with eggs having blastodermic discs.

During the summer in Newfoundland growth of these candle embryos proceeds until by October and November they are from 2 to 4 cm. in length. However, by

the first of October most of the large females have left Newfoundland and it is of interest that at this time they become abundant around Cape Cod and constitute the group we have designated as being in Stage *B* of gestation. Templeman's data based on measurements of the embryos of nine fish during October and November fall well within the range of those for our Stage *B*. The only exception being that we found some embryos that were about 8 cm. in length but at the same time we had an opportunity to examine a much larger number.

Dogfish with pups having large yolk sacs (Stage *C*) taken at Woods Hole in May are comparable with those caught in Newfoundland in July. The length of the pups in Newfoundland varied from 12 to 18 cm. (average 15.59 cm.). A few at Woods Hole were 20 cm. in length, but this is probably an unimportant variation. There is also considerable variation in the size of the yolk sac. These differences in length and size of yolk sac, it seems, do not indicate necessarily a corresponding difference in age but rather is correlated with the size of the ovum at ovulation.

Such young, during the stay of the parent in Newfoundland from July to November, grew in length from an average of 15.59 cm. to 21.43 cm. The average length of the young in November is essentially the same as that for similar young taken at Woods Hole during the same month. However, there were certain interesting exceptions. The largest pups among those measured by Templeman were 25.5 cm. in length, while our largest measured 29.00 cm. The yolk sacs of these large pups had been absorbed almost completely while those of others somewhat smaller were reduced in diameter to about 1.75 to 2.0 cm. There were also a few large females with empty uteri. The large ovarian ova and uteri of these animals indicated that they had given birth to their young just recently. It seems probable that the great majority of births occur at a later time somewhere south of Woods Hole, but in a few instances young are released during the fall migration southward.

#### CONCLUSIONS

The spiny dogfish, *Squalus acanthias*, of the Atlantic coast migrate northward in the spring and southward in the fall. They are present at Woods Hole, Massachusetts, from late April to early June and again in October and November. The adult females when they appear in the spring, have uterine young which are either early embryos enclosed in membranous capsules ("candles") or more advanced "pups" 12 to 20 cm. in length, with large pendent yolk sacs, and free in the uterine lumen. Adult females, when seen in the fall, have candle embryos 3.5 to 7.5 cm. long or large pups 25 to 29 cm. in length, whose yolk sacs are greatly reduced or completely absorbed, while a few females have given birth to their young. Thus four distinct stages of gestation are seen when the fish are divided on the basis of development of uterine young.

The gestation period apparently covers about 20 to 22 months and a female gives birth every other year. This opinion is supported by the progressive advancement in development of the young in the four stages of pregnancy. Also, correlated with this is a gradual involution of the corpora lutea and a corresponding increase in size of the ovarian ova which will be ovulated following the conclusion of the existing pregnancy.

Analysis of candle ova and pups of the four stages of gestation show an increase in water and a decrease in both organic and inorganic matter as indicated by dry

weights and content of ash. Thus it seems that gestation in the spiny dogfish should be considered as being ovoviviparous.

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# THE CULTURE OF VOLVOX AUREUS EHRENBURG

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## INTRODUCTION

A number of methods for the culture of *Volvox* are described in the literature, but none of these has been consistently successful in our laboratory. Other investigators have had a similar experience. S. O. Mast (personal communication) and his students have tried these methods time and again over a period of years without securing permanent cultures.

Hartmann (1921) was unsuccessful in culturing *Volvox* although he was able to maintain cultures of *Eudorina elegans* for many months. Knoke's many experiments (1924) over a two-year period were likewise without positive results. After extensive analyses of natural waters containing *Volvox* in large numbers, Uspenski and Uspenskaja (1925) devised an inorganic salt solution (based on Knop's medium) in which *Volvox aureus* Ehrenberg and *V. globator* Linnaeus were cultured bacterium-free for periods of fifteen and four months respectively. These workers showed that a favorable concentration (0.5–1.0 mg. l.) of iron was a decisive factor in the production of healthy cultures, a deficiency of iron resulting in gradual deterioration, an excess of iron producing a poisoning of the *Volvox* colonies. Soil extract or decoction was used by Mainx (1929) who secured rich permanent cultures of *V. aureus* far superior to those obtained with synthetic solutions, including that of Uspenski and Uspenskaja. Pringsheim (1930) reached similar results in experiments with *V. globator* and *V. aureus*, but he likewise was unable to maintain these organisms for any length of time in the Uspenski medium where their growth was slower and of shorter duration than in soil extract medium. Lefèvre (1932) states that he secured a clone culture of *V. aureus* in a nutritive solution (derived from that of Czurda) consisting largely of inorganic salts in pond water to which a few fragments of *Sphagnum* were added. About 300 descendant colonies were produced in some ten days, but he does not say whether this clone was maintained for any appreciable length of time. Mixed cultures of Volvocales and other plankton species, which included *V. aureus*, *Pandorina morum* Bory, and *Eudorina elegans* Ehrenberg, were maintained for many months.

Johansen (1940) lists six solutions which produce luxuriant growth of *Volvox*, *Gonium*, *Pandorina*, and *Eudorina*, namely certain soil solutions; 0.05 per cent Benecke's and 0.05 per cent Knop's solutions; and 1.5 per cent Detmer's agar (apparently after Bold, 1936). He also gives a medium which is identical in composition with that of Uspenski.

It would appear from the foregoing that the continuous culture of *Volvox* is a relatively simple and exact matter, and that a variety of media are available for this

<sup>1</sup> I gratefully acknowledge the friendly interest and encouragement in this work shown by the late Dr. S. O. Mast, emeritus professor of zoology, The Johns Hopkins University.

purpose. Nevertheless, preliminary experiments indicated that this is not the case. Soil decoctions vary widely in composition with the soil used, and inorganic salt solutions may not give rich, permanent cultures.

The purpose of the present paper is threefold: (1) to describe a culture method for *V. aureus* which has given consistent results for eighteen months in the continuous culture of the organism; (2) to reduce this method to one which is readily duplicable; and (3) to indicate by experiments the probable complexity of the nutrition of this organism.

#### MATERIALS AND METHODS

*V. aureus* was secured from a pond on the property of the Carolina Biological Supply Company near Elon College, North Carolina, where it occurs throughout the year, appearing intermittently in large numbers.

In all of the experiments ordinary finger bowls (4½ inches × 2 inches) were employed as culture dishes. Two hundred cc. of medium were dispensed in each of the bowls which were then placed in a hot air oven and pasteurized. Inoculations were made after the medium had cooled to room temperature (approximately 21° C.). All cultures were illuminated by the light through a west window, some direct sunlight falling on them during part of the afternoon. Throughout the winter months the low light intensity was supplemented by radiation from a Sylvania fluorescent lamp (Type HF-150S).

Inoculations were made with ordinary medicine droppers which had been previously boiled. No attempt was made to exclude bacteria from the cultures, which however showed little evidence of bacterial action. All other possible contaminants such as protozoa and algae were absent.

The salts used in the preparation of inorganic media were either Baker's Analyzed or Merck's Reagent. The peptone and beef extract were Difco, the creatine was Pfanstiehl co., the lactic acid was Baker's Analyzed, and the uric acid was Coleman and Bell's Reagent. In all cases stock solutions were made up and then diluted to proper concentration in the preparation of media.

The commercial fishmeal (commonly used as fertilizer) was obtained from Ballard Brothers, Willis Wharf, Virginia. The spring water used in most of the experiments was secured from a surface spring on the property of the Carolina Biological Supply Company. It is referred to as local spring water. The Huckleberry spring water came from a spring by that name near Durham, North Carolina. Distilled water was supplied by a Stokes Automatic Water Still.

#### EXPERIMENTS AND RESULTS

##### *Experiments with inorganic salt solutions*

Modified Knop's medium as recommended by Bold (1936) and Johansen (1940) was prepared and dispensed into finger bowls. Five cultures were inoculated with several hundred colonies of *Volvox*. The cultures were examined daily, but no growth or reproduction was evident. The colonies gradually lost their rich green color and fell to the bottom of the bowl where they remained motionless, and finally deteriorated. Although the experiment was repeated a number of times the result was always the same.



Since Uspenski and Uspenskaja (1925) cultivated *V. aureus* and *V. globator* in an inorganic medium continuously over periods of fifteen and four months respectively, attempts were made to secure similar results with their medium. The composition is as follows:

KNO <sub>3</sub> .....	0.025 gm.
MgSO <sub>4</sub> .....	0.025 gm.
Ca(NO <sub>3</sub> ) <sub>2</sub> .....	0.100 gm.
KH <sub>2</sub> PO <sub>4</sub> .....	0.025 gm.
K <sub>2</sub> CO <sub>3</sub> .....	0.0345 gm.
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	0.00125 gm. (added every 10 days)
Distilled water up to 1,000 cc.	

Whereas Uspenski and Uspenskaja worked with bacterium-free cultures and used Leningrad glass culture dishes, the present experiments were conducted in ordinary glass finger bowls containing pasteurized medium. The initial pH was found to be 7.6 which is in exact agreement with these authors.

Five cultures were prepared. Each was inoculated with a single large *Volvox* colony bearing eight daughter colonies. Only one culture showed any evidence of growth and reproduction about a hundred pale green colonies being evident after several weeks. These were permitted to concentrate at one side of the bowl, removed with a medicine dropper, and used as inoculum for five subcultures. Observation over a period of four weeks showed no evidence of growth and reproduction. Gradually the colonies lost their motility, became paler in color, and finally disintegrated. Although the experiment was repeated a number of times, the results were negative.

It was noted that the ferric sulfate had a tendency to be precipitated in some of the cultures shortly after they were pasteurized. Believing that failure of the experiment might be due to a lack of the iron salt, it was repeated with the exception that FeCl<sub>3</sub> was used instead of Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>; but, although the former salt remained in solution somewhat better than the latter, the end result was the same.

#### *Experiments with media containing organic materials*

**Soil decoctions.** Several samples of local garden soils were obtained, extracted, and diluted according to the methods of Mainx (1929) and Pringsheim (1930). Although numerous attempts with varied concentrations of soil extract medium were made to secure cultures comparable to those described by these workers, success was not realized. In most instances the organisms rapidly deteriorated and died. In several cases multiplication occurred for a time, but efforts to maintain these cultures through subcultivation failed.

**Peptone, beef extract, milk, and urine.** Various concentrations of these substances in spring water ranging from 5 mg./l. to 200 mg./l. were tested without securing growth and reproduction for more than a week or so. The first cultures of *Volvox* in beef extract medium (5 mg./l.) exhibited an astounding rate of multiplication for several weeks, thousands of colonies being produced. Subcultures, however, were poorer. Gradually the colonies became paler in color, and after six weeks the third set of subcultures died out. Some evidence of growth was secured in peptone medium (5 to 10 mg./l.) but *Volvox* colonies quickly deteriorated in all concentrations of urine and milk media, in which a considerable growth of bacteria

was evident. Even though all of the culture media contained  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (0.5 cc. 1 per cent sol./l.), this failed to prevent the cultures from dying out.

**Commercial fishmeal.** An extract of commercial fishmeal was prepared by adding 200 mg. of fishmeal to a liter of spring water and heating to  $80\text{--}90^\circ\text{C}$ . The mixture was shaken well and filtered (Reeve filter paper, No. 201). The slightly straw-colored filtrate was dispensed in five finger bowls which were then pasteurized. Several hundred *Volvox* were inoculated into each of these. After two weeks one of the cultures was teeming with colonies. Five subcultures from it produced thousands of the organisms. Subcultivation was continued every two weeks, but after six weeks all of the descendant cultures suddenly died out.

This experiment was repeated in exactly the same way except that the cultures were aerated daily by bubbling air through the medium, but the end result was the same.

Recalling that Uspenski and Uspenskaja (1925) had emphasized the importance of iron in the nutrition of *Volvox*, fishmeal extract was prepared as in the previous experiments. To each liter 0.5 cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was added. This gave a concentration of the salt of 5 mg./l. or of 1.03 mg. Fe/l. This amount of Fe is slightly greater than the optimum (0.5–1.0 mg./l.) given by Uspenski and Uspenskaja, but it was felt that some of the Fe would be bound by organic substances in the medium and so reduce the available supply. The amount of Fe remaining unbound would be within the optimal range.

Five cultures were set up and inoculated from a clone culture that had been established in fishmeal extract several weeks earlier. This culture contained no iron other than that already present in the spring water used in its preparation.

In approximately two weeks excellent cultures resulted. From these cultures others were established which were in no way inferior. Thus, this clone of *Volvox* has been maintained in continuous culture with undiminished vigor for a period of eighteen months.

Following the recommendation of Uspenski and Uspenskaja, only half as much iron ( $\frac{1}{4}$  cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) was added in winter as in summer. Since the initial concentration of iron was rather high, iron was not added every ten days in summer and once a month in winter as they suggested. The initial pH of the fishmeal medium was consistently 7.6. Over a three-week culture period it was found to fluctuate around this value.

Fishmeal medium, prior to the addition of ferric chloride, is faintly straw colored but clear. After twenty-four hours it becomes slightly cloudy, perhaps through bacterial action. No membrane forms at the surface, however, and in a few days the medium becomes clear.

No attempt has been made to count the total number of colonies in a single culture, but the number surely runs up to several thousand. Cultures reach a maximum population in from two to three weeks depending largely on variations in light intensity and temperature. Over a period of eighteen months reproduction has been asexual; sexual reproduction has not been observed.

Extracts of different strengths in which the amount of fishmeal varied between 50 and 1,000 mg./l. were tested, but 200 mg./l. seemed to give the largest populations of *Volvox*. Therefore, all stock cultures were maintained in media prepared with that amount of fishmeal.

*Composition of fishmeal extract*

Since *I. aureus* flourished in a medium prepared from spring water, fishmeal, and ferric chloride, it seemed desirable to determine the essential components with a view toward simplification. In this connection the following questions are pertinent: Can a spring water of known chemical composition be substituted for the local spring water? Can spring water be replaced by either tap water or distilled water? Can Uspenski's medium be used in place of spring water? Are the inorganic salts alone in fishmeal, along with those in spring water, sufficient to support the culture of *Volvox*? Does the fishmeal extract contain an appreciable amount of protein, and if so, how important is this in the medium? How important are other extractives?

**Water component of fishmeal extract.** Seven lots of five cultures were prepared in which the following media were used:

1. Fishmeal + local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
2. Fishmeal + Huckleberry spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
3. Fishmeal + tap water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
4. Fishmeal + distilled water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
5. Fishmeal + Uspenski medium
6. Fishmeal + Uspenski medium ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  used instead of  $\text{Fe}_2(\text{SO}_4)_3$ )
7. Local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$

In all cases 200 mg. of fishmeal and  $\frac{1}{2}$  cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  were used.

Each culture was inoculated with ten large *Volvox* colonies of approximately equal size, each colony containing eight daughter colonies.

*Volvox* flourished in media 1 and 2, but little or no growth occurred in 3, 5, 6, and 7. Large numbers of colonies were produced in medium 4. These were pale green in color, becoming paler with each subcultivation, and finally were moribund after four transfers (68 days). Cultures in media 1 and 2 were maintained for many months and were in a flourishing state when finally discarded. Repetition of the experiment gave substantially the same results.

This experiment clearly shows that the chemical composition of the water used in fishmeal medium is very important in the culture of *Volvox*. Tap water may not be nutritively deficient but toxic, for it has been observed that paramecium, hydra, and other invertebrates do not survive long in the local unchlorinated, artesian water. Distilled water is obviously deficient in certain essential salts, although the lack is not felt for some time. Huckleberry spring water can be substituted for the local spring water since it can supply these salts in adequate amounts. It is interesting to note that the Uspenski medium, prepared to include either ferric sulfate or ferric chloride, failed to support growth and reproduction of *Volvox*, even when fortified with the extractives of fishmeal. Finally, fishmeal supplies important nutritive substances without which cultures of *Volvox* soon perish.

**Fishmeal component.** Obviously fishmeal is a very complex material. Therefore, no extensive series of experiments was contemplated to determine what substances in fishmeal are so vital in the nutrition of *Volvox*. The following experiment was performed to make clear what class or classes of substances (inorganic

salts, proteins, other extractives) in fishmeal contribute to the growth and reproduction of *Volvox*. Four culture media were prepared according to the schema:

1. Fishmeal ash + local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
2. Fishmeal + cold local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
3. Fishmeal residue + hot local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
4. Washed fishmeal + local spring water +  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$

One gram of fishmeal was completely incinerated in a chemically clean crucible and the resulting ash shaken well with one liter of spring water. The mixture was heated to 80–90° C. and filtered while hot. Two hundred cc. of this solution were diluted to one liter with spring water in order to secure an inorganic salt concentration comparable to that of regular fishmeal extract.

Another gram of fishmeal was added to a liter of spring water, shaken well, and filtered. Two hundred cc. of the filtrate were diluted with spring water to one liter to give a solution of cold-water extractives approximating the concentration of these substances in regular fishmeal extract.

The residue on the filter paper was added to a liter of spring water and heated to 80–90° C. and filtered while hot. Two hundred cc. of the filtrate were diluted with spring water to one liter. Theoretically this solution contained hot-water extractives roughly equivalent to their concentration in regular fishmeal extract.

The residue on the filter paper was dried and weighed. It had lost one-fourth of its weight. This amount (0.25 gm.) was shaken well with a liter of spring water to form a suspension.

One-half cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was added to each liter of culture medium. Five cultures were prepared from each of the four media, pasteurized, and allowed to cool to room temperature. Each culture was inoculated with five large *Volvox* colonies, every one of which contained eight daughter colonies in about the same state of development.

Examination of the cultures over a period of three weeks gave no evidence of growth in media 1, 3, and 4. Medium 2 on the other hand gave excellent cultures which were in no respect inferior to stock cultures maintained on regular fishmeal extract. Subcultures, which were carried for several months, showed no sign of deterioration whatsoever and probably could have been maintained indefinitely.

This experiment demonstrates, therefore, that the readily soluble cold-water extractives of fishmeal complement the inorganic salts of spring water and the ferric chloride to produce a nutritionally complete medium; that the soluble salts in fishmeal ash medium do not meet all of the growth requirements of *Volvox*; and that hot-water extractives and proteins of fishmeal have little or no importance in the culture of *Volvox*.

It might be supposed that proteins would be present in the cold-water extract fraction of fishmeal, but the usual tests for protein were negative.

### *Fish extracts*

Commercial fishmeal consists largely of the pulverized remains of marine fishes, and therefore contains all of the chemical constituents of bone, muscle, skin, viscera, etc. The question as to what part of a fish, if any one part, provided the important nutritive substances in the culture of *Volvox* was investigated as follows:

Several butter-fish (*Poronotus triacanthus* Peck) were secured at a local fish market. These were washed in distilled water. Small quantities (about 10 gm.) of skin, muscle, and bone (vertebral column) were removed very carefully in order not to include any of the adjacent tissues. The three portions were placed in a small amount of distilled water in three separate flasks and boiled until practically all of the water had evaporated. They were then dried on filter paper in a dry air oven. One-half of each portion was separately shaken with ether from time to time for a period of four hours in order to remove lipoids, after which treatment the ether was removed by filtration. The skin, muscle, and bone residues were dried on filter paper for forty-eight hours. The remaining half of each portion of skin, muscle, and bone received no further treatment.

Two hundred mg. each of skin, skin extracted with ether, muscle, muscle extracted with ether, bone, and bone extracted with ether were separately heated with one liter of spring water to 80 – 90° C. and filtered while hot. One-half cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was added to each liter of filtrate. Five cultures were prepared from each type of filtrate and inoculated with numerous *Volvox*.

The results are summarized below:

1. Skin medium—colonies died within a few days
2. Extracted skin medium—colonies died within a few days
3. Muscle medium—good cultures; subcultured for three months
4. Extracted muscle medium—excellent cultures; subcultured for three months
5. Bone medium—colonies died within three weeks
6. Extracted bone medium—some growth and reproduction; subcultures unsuccessful

The experiment was repeated using Huckleberry spring water instead of the local spring water. The results were essentially the same with the exception that colonies in the extracted bone medium died out within a week.

The experiment shows that, of the three tissues tested, muscle alone contains an adequate quantity of the substances so vital to the continuous culture of *Volvox*. Furthermore, the conclusion is reached that the lipoids are of no value to *Volvox*; on the contrary they appear to exert a depressing influence as is indicated by the larger populations of extracted muscle medium cultures.

No attempt is made here to deny that connective tissue associated with fish muscle may contribute as much or more than muscle to the nutrition of *Volvox*. It would be next to impossible to separate the two tissues to determine this point. However, in experiments which follow the assumption is made that it is the muscle that is important and not the connective tissue.

Since the butterfish is marine and the associated salts of sea water might conceivably have something to do with the results obtained, experiments were carried out with the fresh-water sunfish (*Lepomis gibbosus* Linnaeus) in order to check this presumption. The ether-extracted, powdered muscle gave flourishing cultures which were maintained in subculture for three months and then discontinued. Doubtless muscle tissue from other fishes could have been used with similar results.

#### *Experiments with some organic constituents of fish muscle*

Whatever the nature of the constituents of fish muscle that are so important in the culture of *Volvox*, it is clear from the foregoing experiments that the proteins,

lipoids, and inorganic salts are unnecessary. This leaves carbohydrates (glycogen, etc.), nitrogenous extractives (creatine, urea, uric acid, etc.), non-nitrogenous extractives (lactic acid, inositol, etc.), pigments, enzymes, and growth-promoting factors, and perhaps other substances to be considered. It seemed most likely that one or more of the nitrogenous and non-nitrogenous extractives might support the continuous culture of *Volvox*. Therefore, experiments were undertaken in which lactic acid, urea, creatine, and uric acid were tested singly and in combination in various concentrations from 5 mg./l. to 100 mg./l. of local spring water, the usual amount of ferric chloride being added to all media. None of the cultures, however, was successful. In several instances adult colonies released daughter colonies, but these gradually lost their healthy green color and soon deteriorated. In a medium composed of urea, peptone, and lactic acid (5 mg./l. each) as many as three generations were produced before death occurred.

#### *A duplicable culture medium for Volvox aureus*

An analysis of Huckleberry spring water by D. M. Pace some fifteen years ago (personal communication) showed it to have the following inorganic composition:

Na <sub>2</sub> SiO <sub>3</sub> .....	12 mg./l.
NaCl.....	12 mg./l.
Na <sub>2</sub> SO <sub>4</sub> .....	6 mg./l.
CaCl <sub>2</sub> .....	6.5 mg./l.
MgCl <sub>2</sub> .....	3.5 mg./l.
FeCl <sub>3</sub> .....	2-3 mg./l.

An artificial spring water was made up according to the above formula except that sufficient ferric chloride solution was added to give a concentration of this salt of 5 mg./l. Using ether-extracted, powdered sunfish muscle (200 mg./l.), culture medium was prepared according to the same method previously described for the fishmeal and fish muscle media. Cultures of *Volvox* have been maintained on this medium in a flourishing state for a period of four months, showing no evidence of decrease in vitality. These cultures were in every respect equal to cultures of the organism secured with commercial fishmeal extract.

Although fishmeals may vary somewhat in composition depending on their source and mode of manufacture, the powdered sunfish muscle prepared as herein described may be expected to have a constant composition wherever prepared. This method for the culture of *V. aureus*, therefore, is readily duplicable.

#### DISCUSSION

Results of the foregoing experiments fail to confirm those of Uspenski and Uspenskaja (1925), Bold (1936), and Johansen (1940) who have cultured *Volvox* in inorganic salt solutions in the absence of organic matter. The findings, however, are in agreement with those of S. O. Mast (personal communication) and his students who were unable after many experiments to secure permanent cultures of *Volvox* in Uspenski's, Knop's, and other synthetic media. Pringsheim (1930) was evidently unable to maintain *V. aureus* and *V. globator* for any length of time in the Uspenski medium.

It is difficult to believe that failure to culture *Volvox* in the Uspenski medium was a result of impurities in the salts or the kind of glassware used in the present experiments. The salts were of the same degree of purity as those generally employed in the culture of protozoa and algae. The salts used in the preparation of artificial Huckleberry spring water apparently had no toxic effect.

Various investigators, notably Pringsheim (1930), have called attention to the deleterious effects produced on organisms by the kind of glass of which culture dishes are composed. He believed that unfavorable results were due to soluble constituents of the glass causing the medium to become more alkaline. Although ordinary finger bowls were used in the present experiments, no deleterious effect of the glass was observed. Successful cultures on fishmeal and fish muscle extract media were secured in the same glass finger bowls in which Uspenski's medium failed to support continuous growth and reproduction of *Volvox*.

Nor can failure to secure permanent cultures in the Uspenski medium be ascribed to an unfavorable pH. The initial pH was 7.6 (identical with that given by Uspenski and Uspenskaja) and remained at that value over a period of three weeks. That this is a favorable reaction is shown by the fact that the pH of freshly prepared fishmeal extract medium is 7.5-7.6, around which value it fluctuates from day to day gradually rising to a final pH of 8.0-8.2.

Mainx (1929) and Pringsheim (1930) secured rich cultures of *Volvox* in decoctions prepared from garden soil. Soils, however, vary widely in composition and reaction so that this method is not readily duplicable. Fishmeal medium, on the other hand, can be made up fairly accurately and is more readily reproducible. This is even more true of the fish muscle extract prepared with artificial Huckleberry spring water and the muscular tissue of the sunfish or butterfish.

The experiments of the present work show that the nutritional requirements of *Volvox aureus* may be more complex than is indicated by the work of Uspenski and Uspenskaja (1925) and that inorganic salts of spring water as well as organic substances of fish muscle are essential if this organism is to be maintained in culture for any length of time. What these salts and organic substances are, remains for further work to show; but it is quite possible that water-soluble growth promoting substances play an important rôle.

#### SUMMARY

1. *Volvox aureus* Ehrenberg has been maintained in rich clone cultures for eighteen months without observable decrease in vitality on a medium prepared from spring water, commercial fishmeal, and ferric chloride.

2. The presence of iron in the medium was found to be essential to the continuous culture of *Volvox*. This was added as ferric chloride. Cultures lacking iron died out in about six weeks.

3. Either local spring water or Huckleberry spring water served satisfactorily in the preparation of fishmeal extract medium, but tap water and distilled water could not be substituted for these.

4. Spring water media containing peptone, beef extract, milk, or urine in various concentrations would not support continuous growth and reproduction of *Volvox*.

5. Uspenski's medium and modified Knop's solution were found to be of little value in the culture of *Volvox*.

6. Powdered muscle of the marine butterflyfish (*Poronotus triacanthus* Peck) or the fresh-water sunfish (*Lepomis gibbosus* Linnaeus), with lipoids extracted, could be substituted for commercial fishmeal, while skin and bone could not.

7. Spring water containing only the inorganic salts of fishmeal, or only the proteins, failed to support the culture of *V. aureus*; but spring water containing the cold water extractives and ferric chloride (one-half cc. of a one per cent solution of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ /l.) gave excellent cultures which probably could have been carried indefinitely.

8. *V. aureus* could not be cultured in media containing lactic acid, urea, creatine, uric acid, or a combination of urea, peptone, and lactic acid in the several concentrations used.

9. A readily duplicable method for the culture of *V. aureus* is described.

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# OXYGEN CONSUMPTION AND CARBON DIOXIDE ELIMINATION IN *TETRAHYMENA GELEII* FURGASON<sup>1</sup>

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Unicellular organisms make excellent material for the study of various cellular phenomena. *Tetrahymena geleii*, a colorless holotrichous ciliate, is an exceptionally desirable organism for such physiological studies, mainly because it is readily grown in rather simple, sterile organic media.

This organism contains cytochromes c, b, a, and possibly a<sub>2</sub> and its oxygen consumption is inhibited by cyanide and carbon monoxide (Baker and Baumberger, 1941). It grows well at ordinary oxygen tensions, but is most prolific in pure oxygen (Pace and Ireland, 1945). It soon dies in oxygen tensions below 10 mm. Hg partial pressure.

Many investigations have been carried out with *Tetrahymena* as the experimental organism. There is, however, much to be desired in respect to our knowledge concerning respiratory metabolism; knowledge that may lead to a better understanding of the respiratory mechanisms of cells in general. Since very little is known concerning oxygen consumption, carbon dioxide elimination, and respiratory quotient in *Tetrahymena*, the following investigations were made.

## MATERIAL AND METHODS

The "W" strain of *Tetrahymena geleii*, kindly furnished by Professor George Kidder, used throughout these investigations, was grown in this laboratory in a 2 per cent proteose-peptone (Difco) solution. They grow very rapidly in this solution and usually reach maximum numbers within four to six days depending upon temperature and the number introduced.

A Barcroft-Warburg respirometer was used for ascertaining oxygen consumption and carbon dioxide elimination. Temperatures below room temperature were obtained in the bath by means of an Aminco refrigerating unit.<sup>2</sup>

Before each experiment the organisms were washed thoroughly in a buffered solution in which they were left during the experiment. This solution is one similar to that used by Pace and Belda (1944) for *Pelomyxa*, with slight modifications,<sup>3</sup> and was used for these tests chiefly because of the several advantages it has over the proteose-peptone solution. It is much simpler to work with since there is no

<sup>1</sup> Aided by a grant from the Penrose Fund of the American Philosophical Society.

<sup>2</sup> An extra set of manometers and shaking device for the Warburg outfit was purchased for this investigation by a grant-in-aid furnished by the University of Nebraska Research Council.

<sup>3</sup> The buffer solution used for washing and testing *Tetrahymena geleii* in the Barcroft-Warburg respirometer contained the following: K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 65.5 mg.; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 40.0 mg.; CaCl<sub>2</sub>, 100 mg.; MgCl<sub>2</sub>, 2.0 mg.; and redistilled H<sub>2</sub>O to 1,000 ml.

food material on which bacteria can grow and therefore the dangers of contamination are minimized. Bacteriological technique was used throughout.

Usually, for each condition tested, two different ages of cultures were used: "young" cultures (three or four days) and "old" cultures (seven or eight days). These cultures were started by introducing several thousand organisms by means of a platinum wire loop into 70 ml. of sterile proteose-peptone solution in a 125 cc. pyrex erlenmeyer flask.

After washing and after proper dilution or concentration (by centrifugation) 5 ml. of culture solution of known population density were added to each Barcroft-Warburg flask except to the thermobarometer which contained 5 ml. of buffered solution without organisms.

Oxygen consumption and  $\text{CO}_2$  elimination were determined by the direct method. For this purpose 0.2 ml. 10 per cent KOH was put into the inner wells (insets) of one-half the flasks and 0.2 ml.  $\text{H}_2\text{O}$  in the other one-half. To each of the onsets was added 0.3 ml. 3N  $\text{H}_2\text{SO}_4$  to absorb any ammonia that might possibly be formed in metabolism. In every case this acid was dumped into the main compartment of the flask at the end of an experiment after which a reading was always made in order to account for the bound  $\text{CO}_2$  which is released by the action of acid.

## RESULTS

### *Relation between population density and $\text{O}_2$ consumption and $\text{CO}_2$ elimination*

Tetrahymenas were obtained from cultures of different ages. "Young" cultures were produced in the following manner: about  $2,000 \pm 200$  tetrahymenas were added to 70 cc. 2 per cent sterile proteose-peptone solution in pyrex erlenmeyer flasks. These cultures were kept at room temperature ( $24^\circ \pm 2^\circ \text{C.}$ ) for 3 or 4 days when the organisms were washed in the buffer solution\* by centrifugation. They were then ready for testing. Several different population densities were used, ranging from approximately 10,000 to 195,000 organisms per ml. Several experiments were conducted for each density.

In these experiments 5 ml. of fluid containing young tetrahymenas were added to each of six Barcroft-Warburg flasks (3 with KOH in inner well; 3 with  $\text{H}_2\text{O}$ ). Observations and readings were made from time to time during the course of the experiment. Usually readings were made every hour, but in the greater population densities, they had to be made every 15 or 30 minutes; whereas, with some of the lower population densities several hours elapsed in some cases before a final reading was made. It depended entirely upon the rapidity of oxygen consumption at  $25^\circ \text{C.}$  After the reading was made at the end of an experiment, the acid in the side arm of each flask was dumped into the fluid containing the tetrahymenas. The shaking apparatus was turned on for 10 minutes, after which time another reading was made. The results of these tests are presented in Table I.

The oxygen consumption per organism, when young cultures were used, is greatest in densities of 13,000 to 33,000 organisms per ml. which were the lowest densities used, and least in the greatest densities. In other words, at least within these limits, oxygen consumption per organism is inversely proportional to population density. The respiratory quotient varied from 0.95 to 1.13 in all the different tests, except those in which the lowest population densities were used where the R.Q. averaged 1.41.

The "old" organisms were produced in the same way as the "young" except that they were not used until 7 or 8 days after starting the cultures. The same procedures were followed as in the previous tests. The population densities varied between 12,000 and 265,000 organisms per ml. These results are also presented in Table I.

TABLE I

*Oxygen consumption, carbon dioxide elimination and respiratory quotient in "young" and "old" cultures of Tetrahymena geleii with different population densities. Temperature 25° C.; average volume of one million organisms = 23.6 mm.<sup>3</sup>; 42,370 organisms equal to one cubic millimeter.*

No. of organisms per ml.	Age of culture in days	No. of tests	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per million organisms	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per million organisms	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	R.Q.
Organisms from "young" cultures							
13,000-19,000	3	6	372	15.7	526	22.3	1.41
30,000-33,000	3	9	372	15.7	356	15.0	0.95
41,500-51,500	4	6	325	13.7	350	14.8	1.07
88,000-98,000	3	6	322	13.6	328	13.9	1.01
107,600-121,000	3½-4	9	252	10.6	249	10.5	0.99
195,000	3½	3	246	10.4	278	11.7	1.13
Organisms from "old" cultures							
12,000-12,600	7-8	9	198	8.4	221	9.3	1.11
24,000-27,500	7-8	6	223	9.4	258	10.9	1.15
38,000-39,400	7-8	9	204	8.6	247	10.4	1.21
69,000-72,600	7	6	278	11.7	357	15.1	1.28
87,600-95,000	7-8	6	217	9.2	263	11.1	1.21
135,000-148,700	7	9	207	8.7	240	10.1	1.15
213,000-265,000	7	6	230	9.7	272	11.5	1.18

The oxygen consumption varies from one population density to another much more so than in the "young" cultures, but there is a definite increase in consumption up to approximately 70,000 organisms per ml. and then a decrease with further increase in numbers per unit volume. Carbon dioxide elimination was also ascertained and the R.Q. was found to be greater than unity in every case where old cultures were used. The R.Q. increased progressively from 1.11 when approximately 12,000 organisms per cc. are used, to 1.28 when approximately 70,000 are used. It then decreases with further increase in cell population until it is 1.18 in cultures containing between 213,000 and 265,000 organisms per ml.

#### *Relation between temperature and oxygen consumption*

Tests were made to ascertain the oxygen consumption and carbon dioxide elimination in Tetrahymena at different temperatures. The temperatures used varied from 10° to 35° C. (with 5° increments). As in the previous experiments tests were run on both "young" and "old" organisms. The results are given in Table II.

There is an increase in oxygen consumption as temperatures increase up to 25° C. Above 25° C. the O<sub>2</sub> consumption decreases directly with increase in temperature. This applies to organisms obtained from both young and old cultures. Attempts were made to ascertain oxygen consumption at 40° C. but the organisms died at this temperature. The carbon dioxide elimination and hence the R.Q. values were high in all the tests made. The latter ranged between 1.05 and 1.39.

TABLE II

*The effect of temperature on oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii. Average volume of one million organisms, 23.6 mm.<sup>3</sup>; 42,370 organisms equal to one cubic millimeter.*

Temperature, ° C.	No. of organisms per ml.	Age of culture in days	No. of tests	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per million organisms	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per million organisms	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	R.Q.
Organisms from "young" cultures								
10	31,800	3	3	50	2.1	57	2.4	1.14
15	52,900-67,500	3-3½	9	125	5.3	142	6.0	1.13
20	38,750-41,160	3	6	193	8.1	227	9.6	1.17
25	41,500-51,600	4	6	325	13.7	350	14.8	1.07
30	32,000-60,000	3	6	227	9.6	297	12.5	1.30
35	10,000	3	3	215	9.1	287	12.1	1.33
Organisms from "old" cultures								
10	54,000	8	3	25	1.0	35	1.5	1.39
15	53,000-73,300	7	12	92	3.9	91	3.8	0.99
20	65,500	6-7	6	128	5.4	135	5.7	1.05
25	69,000-72,600	7	6	278	11.7	357	15.1	1.28
30	45,000-50,800	7	6	228	9.6	285	12.0	1.25
35	16,800-19,160	8	6	254	10.7	287	12.1	1.12

*O<sub>2</sub> consumption and CO<sub>2</sub> elimination in Tetrahymena in proteose-peptone solution*

All the previous tests which have been reported here were made on Tetrahymena in the buffer solution given in footnote 3. None had been made on organisms in solutions in which they had grown and lived, i.e., proteose-peptone solution. The following experiment was carried out in order to make these determinations. Thus, the tests were conducted in the same way as the preceding ones, except that the organisms (1) were kept in the solution in which they had grown (2 per cent proteose-peptone) or (2) were washed in fresh proteose-peptone and then put into the manometer flasks in this solution. It was found that the hydrogen-ion concentration did not hold to a constant value as well as in the buffer solution but the change did not seem to harm the organisms (the greatest change noted was a change from pH 6.8 to pH 7.3). The results are presented in Table III.

In the tests in which "young" cultures of Tetrahymena were used in fresh proteose-peptone solution, the oxygen consumption proved to be much greater than in those tested in buffer solution (at 15° C., 271 mm.<sup>3</sup> compared to 125 mm.<sup>3</sup> per hour

per million organisms in buffer solution; at 25° C., 666 mm.<sup>3</sup> compared to 325 mm.<sup>3</sup>). In the organisms from "old" cultures, however, the oxygen consumption was somewhat lower than that in buffer solution (at 15°, 70 mm.<sup>3</sup> as compared to 92 mm.<sup>3</sup>). Variance in numbers, however, must be taken into consideration.

TABLE III

*Oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii in 2 per cent proteose-peptone solution. Temperature varied as indicated; average volume of one million organisms, 23.6 mm.<sup>3</sup>; 42,370 organisms equal to one cubic millimeter.*

Temperature, °C.	No. of organisms per ml.	Age of culture in days	Condition of proteose-peptone solution	No. of tests	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per million organisms	O <sub>2</sub> consumption in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per million organisms	CO <sub>2</sub> elimination in mm. <sup>3</sup> per hr. per mm. <sup>3</sup> cell substance	R.Q.
15	30,400	3	fresh	4	271	11.4	328	13.9	1.21
15	32,060–40,000	7	old	6	70	2.9	197	8.3	2.81
25	25,800	3	fresh	3	666	28.2	849	35.9	1.27

A very interesting observation brought out by these results, is that in all tests, the R.Q. is considerably above 1.0. This is especially so in the case of the "old" organisms where the average R.Q. for all tests was 2.81.

#### *Significance of the high respiratory quotients*

The question arises as to the true meaning of these high respiratory quotients. In all but a few experiments covered by this report, they are greater than 1.0. It is generally known that high values for R.Q. may be obtained chiefly under two conditions: (1) when fat is in the process of formation in cells or tissues, or (2) when it is necessary for the cell or tissue to go into oxygen debt, temporarily at least, during which time the ratio between CO<sub>2</sub> eliminated and O<sub>2</sub> consumed would be much greater than usual.

*Tetrahymena* produces fat in the form of small globules located in the anterior half of the organism. Observations were made upon these organisms under various conditions in order to ascertain whether or not respiratory quotients varied and if so, whether or not fat content varied also. As far as could be seen by the methods used there was no difference in fat content in organisms under different conditions even though the respiratory quotients varied considerably.

A few tests were made in an attempt to ascertain the oxygen consumption and carbon dioxide elimination of *Tetrahymena* in nitrogen. The nitrogen used, however, was a commercial grade and hence traces of oxygen were present. Although the results obtained by these tests were not conclusive, they indicate that these organisms are anaerobic, at least to a limited extent, thus confirming the results of Thomas (1942). The respiratory quotients calculated from the results ranged between 1.49 and 2.87.

#### DISCUSSION

Hall (1938, 1941), Baker and Baumberger (1941) and Ormsbee (1942) have studied respiration in *Tetrahymena geleii*. The results of these several investiga-

tions appear to differ, in some cases, considerably but when they are analyzed carefully the differences, for the most part, may be explained.

Some of the results of the present investigation are in close agreement with those of the aforementioned workers. This is especially true, when our results are compared with those of Ormsbee (1942) in which he found that the  $O_2$  consumption for these organisms in 2 per cent proteose-peptone solution is 632.5 mm.<sup>3</sup> per hour per million at 26.8° C.; in our studies, it was found that the  $O_2$  consumption in 2 per cent proteose-peptone is 666 mm.<sup>3</sup> at 25° C. The results obtained in non-nutrient media should not be compared since the salts differ greatly in the solutions used by different investigators, but it is found that even in these cases there is some agreement, especially when all the factors are taken into consideration. An explanation of some of the differences, for example, might be found in the fact that different population densities were used. According to our results, as the density of population increases the rate of oxygen consumption per individual decreases. This observation, along with possible differences in age of cultures, and also the difference in culture media before and during the experiment, could account for the slight difference in oxygen consumption as noted in these reports. This age difference is especially noticeable when the organisms are tested in the proteose-peptone solution in which they were grown. There is not only an inhibition of division with age but also a great decrease in oxidative metabolism.

Thomas (1942) showed definitely that *Tetrahymena* is adapted, at least partially, to an anaerobic existence, recovery being dependent upon oxidative metabolism. He believes the processes involved may be similar to those of mammalian striated muscle. Pace and Ireland (1945) showed that *Tetrahymena* can live and grow at very low oxygen tensions but that its growth is best at high tensions. This organism does not grow or live for any great length of time in the total absence of oxygen. Results presented in this report confirm those of Thomas in this respect.

The respiratory quotients obtained for *Tetrahymena* were all high under the conditions in which they were determined. They were highest of all in those organisms confined in practically pure nitrogen. Carbon dioxide is produced in large quantities. It is possible that the metabolism is similar to that of vertebrate striated muscle. The extent of anaerobiosis is limited for *Tetrahymena* just as it is for muscle. *Tetrahymena* can live for several days in the absence of oxygen but soon dies unless oxygen is added. Specht (1934) found the same to be true for *Spirostomum*. He obtained high R.Q. values when this organism was exposed to low  $O_2$  tensions. Many protozoan forms function in the same way (von Brand, 1946).

#### SUMMARY

1. Oxygen consumption and carbon dioxide elimination of *Tetrahymena geleii* has been ascertained for different temperatures, for different population densities, and for "young" and "old" cultures.
2. The tests were conducted in most of the experiments with washed organisms in inorganic buffer solution.
3. When "young" organisms are used, the oxygen consumption per unit volume of cell substance is inversely proportional to population density. The respiratory quotients for all tests were nearly always above 1.0.
4. When "old" organisms are used there is an increase in  $O_2$  consumption per unit volume of cell substance with an increase in population density up to 69,000-

72,600 organisms per ml. Densities above this result in lower consumptions. Respiratory quotients varied from an average of 1.11 in low population densities to 1.28 at optimum densities.

5. Oxygen consumption is always greater in the "young" cultures than in the "old" cultures, densities being equal.

6. With an increase in temperature, the  $O_2$  consumption increases to a maximum at 25° C. in both "young" and "old" cultures. Above this temperature the consumption decreases. In all tests, from 10 to 35° C., R.Q. values were above 1.0.

7. "Young" organisms, tested in fresh solution of the same kind in which they were grown, 2 per cent proteose-peptone (Difco), show much greater  $O_2$  consumption than those tested in inorganic buffer solution. "Old" organisms, however, tested in the same solution in which they had grown, have a lower  $O_2$  consumption than old specimens tested in fresh inorganic buffer solution.

8. Very high respiratory quotients were obtained for the organisms in proteose-peptone, especially in "old" cultures where the average was 2.81.

9. In nitrogen gas with but minute traces of oxygen, *Tetrahymena* utilizes the small quantity present and gives off comparatively large quantities of carbon dioxide. The R.Q. values are extremely high.

10. *Tetrahymena geleii* is to a limited extent anaerobic but it can live only with difficulty for more than a few days in the absence of oxygen.

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# INFLUENCE OF TEMPERATURE ON THE ASPHYXIATION OF YOUNG GOLDFISH (*CARASSIUS AURATUS* L.) UNDER VARIOUS TENSIONS OF OXYGEN AND CARBON DIOXIDE

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## INTRODUCTION

In their preliminary work on the sensitivity of Ontario fishes to carbon dioxide as measured by the asphyxial tension of oxygen of fish in sealed jars containing various concentrations of dissolved carbon dioxide, Fry and Black (1938) observed a difference in the response of two sample groups of the minnow, *Chrosomus eos*. The group taken in the autumn from a cold stream near Toronto was found to be appreciably more sensitive than the group from a bog lake in Algonquin Park at the height of the summer warming. These data were subsequently published in a review (Fry, 1939). At that time there was no indication as to the cause of this difference since the two groups came not only from different parts of the country but also from different habitats.

Further measurements were made in Algonquin Park in 1939 when the work was continued into early fall. Some comparisons were made of curves showing sensitivity to carbon dioxide for the same species from the same locality at different times of year. The same effect was noted as for *Chrosomus eos*, although the data were rather scanty. Observations of the same sort were made again in 1940 and, in particular, material was gathered for the brown bullhead (*Ameiurus nebulosus*). The results are illustrated in Figure 1. There is an increased sensitivity in late summer or early fall when the water is cooling. These tests were carried out in water of the same temperature as that in which the animals were captured.

Reasoning by analogy from the fact that cold water species such as the trout fail to take up oxygen in the presence of much lower tensions of carbon dioxide than do warm water species such as the brown bullhead, it was concluded that probably there would be some such trend within each species and that concomitant with the process of thermal adaptation there might be a change in respiratory sensitivity in the direction observed.

Meanwhile an opportunity to test this experimentally had presented itself in February 1939, when two of the authors in collaboration with Professor Laurence Irving and using the facilities of the experimental hatchery at Cornell University, measured the respiratory sensitivity of the speckled trout at 1° C, the temperature of the hatchery water at that time. The respiratory sensitivity was also measured of a group of these fish which had spent 24 hours at 13° C. The sensitivity of the group subjected to the warm water for even that short period of time was appreciably decreased.



This experiment, however, was only of a preliminary nature and at that time it was not possible to carry it to its logical conclusion. For this reason we undertook to do a further series of experiments to determine the effect of temperature on the respiratory sensitivity of thermally adapted goldfish. The temperatures chosen covered the biokinetic range of temperature for this species. Goldfish were maintained at each temperature level for a period of time quite sufficient to ensure a thorough thermal adaptation. The goldfish was chosen because of its suitability as a laboratory animal, because the thermal tolerance for the young goldfish had been

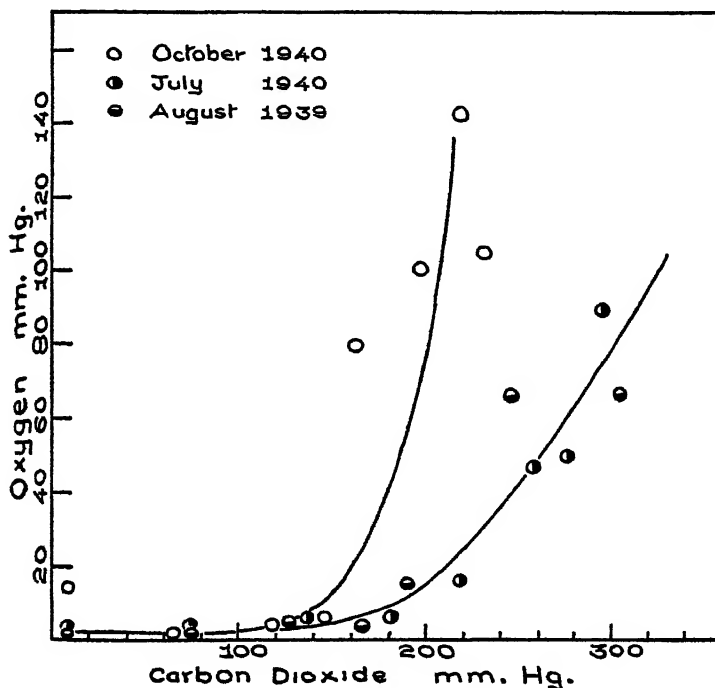


FIGURE 1. Seasonal variation in the extent to which the brown bullhead can reduce the oxygen content of water in sealed bottles in the presence of various tensions of carbon dioxide. In July and August the fish were acclimated to approximately 20° C., in October to 13° C. They were tested at the acclimation temperatures in each instance.

plotted with some exactitude (Fry *et al.*, 1942), and also because the rate of thermal adaptation of goldfish was being measured (Brett, 1946) at the time these experiments were begun.

#### MATERIAL AND METHODS

The goldfish, *Carassius auratus* (Linnaeus), were the ordinary long finned commercial variety of goldfish purchased from commercial dealers in Ontario. They were approximately one year old, of both sexes, and varied from 2½ to 4 inches in length. The fish were fed fox chow during the period of acclimation.

A group of fish was acclimatized to one of the following temperatures: 1° C., 7° C., 15° C., 20° C., 25° C., and 32° C. The overall allowance for acclimation of each

group of fish from 7° to 32° was 1° C. or less per day until the desired temperature was reached. Since complete acclimation to low temperatures takes a longer time (Doudoroff, 1942) the fish used for the 1° C. curve were put from a 7° C. tank into a 1° C. tank and left for two months.

When acclimation was complete each fish was put in a bottle of water (275 ml. capacity) of the same temperature and containing a known tension of carbon dioxide and at least 150 mm. tension of oxygen. During the experiment the sealed bottles containing the fish were kept in a water bath at the temperature of acclimation. When all respiratory movements of the fish had ceased the water in the bottle was analyzed for free carbon dioxide and oxygen. The tension of carbon dioxide was determined as follows: a small bubble of air (0.1–0.3 ml.) was equilibrated with 35 ml. of the water for five or more minutes at the temperature of the experiment at room pressure; the bubble was then transferred to a micro-gas-analyzer (after Krogh, 1908) and the carbon dioxide content of the gas phase was found by absorption of carbon dioxide with  $\frac{1}{4}$ N potassium hydroxide. The tension was obtained by multiplying the fraction of carbon dioxide in the gas phase by the barometric pressure minus the vapor pressure of the water at the temperature of the experiment.

Dissolved oxygen was determined by the unmodified Winkler method, using a 50 ml. sample of water. The pressure of oxygen was calculated by relating the quantity of oxygen found to the solubility at the temperature of the experiment and multiplying the fraction obtained by 760.

## RESULTS

The data for the experiments at each of the six temperatures (1° C., 7° C., 15° C., 20° C., 25° C., and 32° C.) are given in Table I and illustrated in Figure 2. To

TABLE I

*The extent to which goldfish acclimated to various temperatures can reduce the oxygen content of water containing dissolved carbon dioxide. Each pair of values is the mean of three to five determinations*

1° C.		7° C.		15° C.		20° C.		25° C.		32° C.	
CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg
20	5.5	27	3.5	17	2.9	34	7.5	25	4.7	14	8.0
64	4.9	79	3.5	66	3.5	88	8.3	141	13	144	8.0
87	48	100	5.8	110	5.9	120	2.9	172	7.6	168	7.0
98	24	113	16	131	12	139	7.3	200	42	186	7.3
111	46	128	38	144	49	154	41	224	120	212	46
141	66	138	66	153	49	166	25	286	272	226	102
				171	74	197	80			240	216
				192	75	242	139				
				237	163						

reduce the spread the original figures have been grouped into averages of 3 to 5 determinations. This averaging was carried out by arranging all the values in a particular series in order of the magnitude of the carbon dioxide tension and then averaging the values for consecutive groups.

The course at each temperature appears to be as follows. Up to a certain tension of carbon dioxide the oxygen in the bottle is reduced to a uniform and low level before death of the fish ensues. Above a certain carbon dioxide tension the level of the carbon dioxide influences the level to which the oxygen can be reduced, i.e., the greater the carbon dioxide content the higher the level of oxygen in the water at

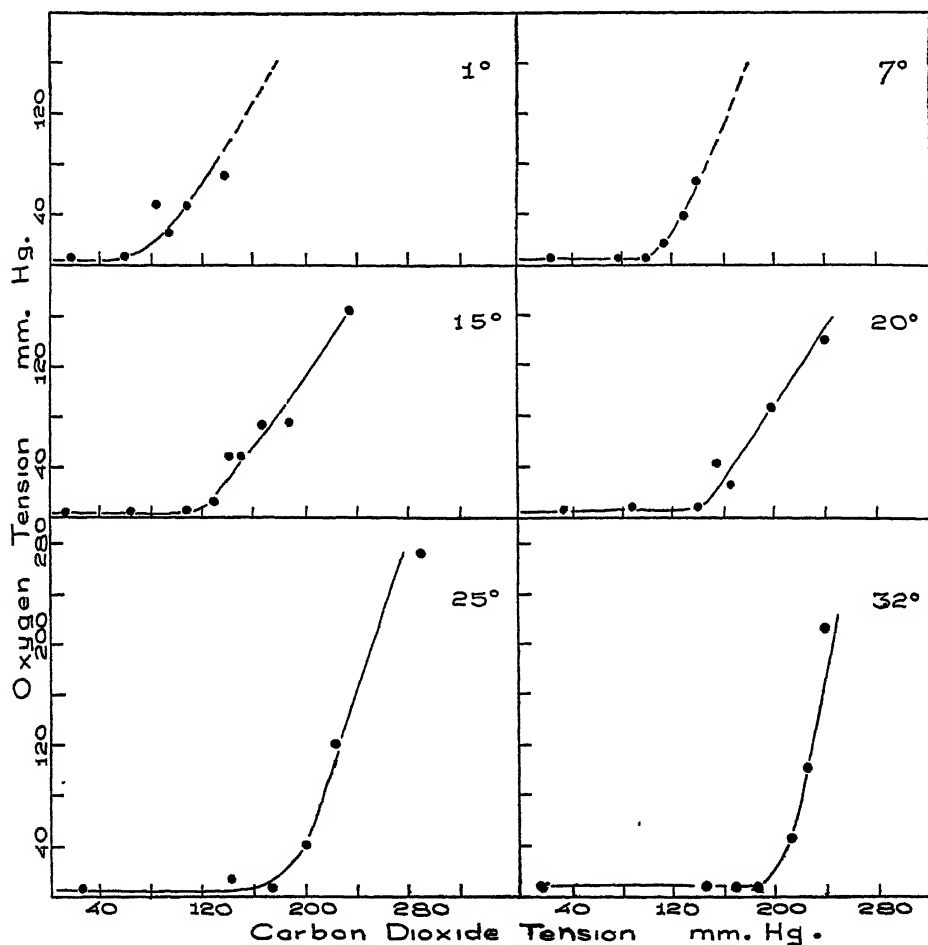


FIGURE 2. The extent to which goldfish can reduce the oxygen content of water in sealed bottles in the presence of dissolved carbon dioxide at various temperatures of acclimation. Each point is the average of three to five determinations.

asphyxiation. As the acclimation temperature is increased the level of carbon dioxide required to limit the level to which the oxygen is used also increases. There is some indication that the rising portion of the curve becomes steeper at the higher acclimation temperatures.

The data from the six curves in Figure 2 are brought together in Figure 3 where the change in sensitivity, i.e., the number of arbitrary units contained within

each curve, is plotted against the temperature of acclimation. The sensitivity of the fish seems to decrease almost directly with the increase in temperature of acclimation.

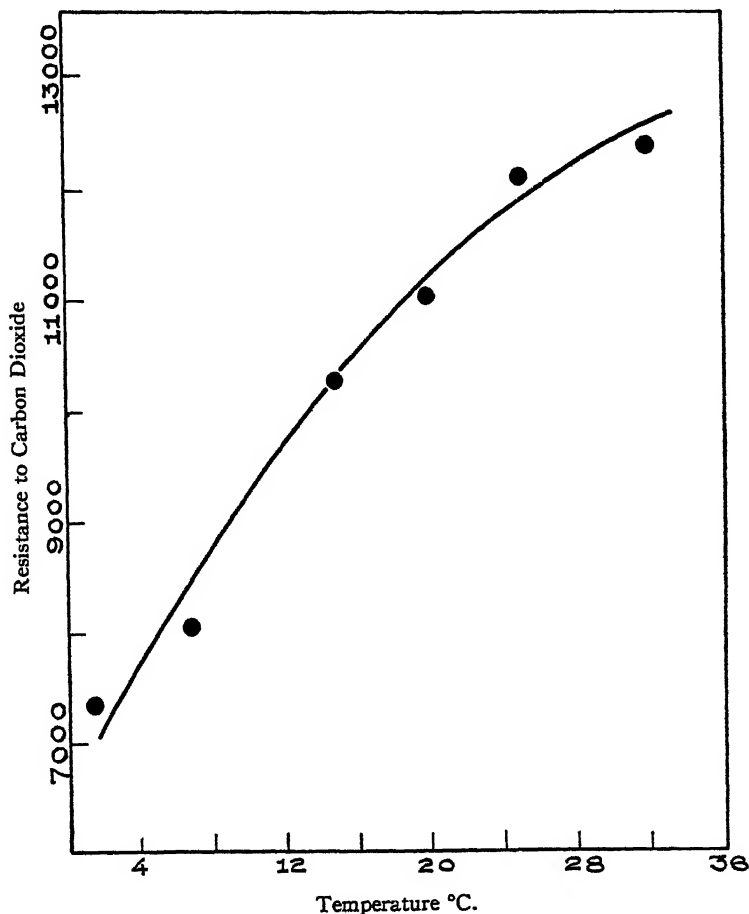


FIGURE 3. The relation between utilization of oxygen in the presence of dissolved carbon dioxide and the temperature to which goldfish are acclimated. The units of resistance to carbon dioxide given are the areas composed by oxygen  $\times$  carbon dioxide (as mm. Hg) between the resistance curves in Figure 2 and a horizontal line drawn across at 160 mm. Hg oxygen (air saturation). Sensitivity to  $\text{CO}_2$ , referred to in the text is a reciprocal function of resistance.

#### DISCUSSION

The ability of the fish to use oxygen in the presence of carbon dioxide as measured here, varies considerably in goldfish adapted to various temperatures within their biokinetic range. The difference between goldfish acclimated to water of 1° C. and those acclimated to water of 32° C. is of the same order as the differences between the common sucker (*Catostomus commersonnii*) and the brown bullhead

(*Ameiurus nebulosus*) when these species are acclimated to about 20° C. (Fry, 1939).

The change in the respiratory sensitivity attendant on change in the environmental temperature is such that when goldfish are adapted to low temperatures their respiratory sensitivity tends toward the type found in cold water species (common sucker): when adapted to high temperatures the respiratory sensitivity resembles that of warm water species (brown bullhead).

Other changes in the respiratory metabolism of fish have been found to be attendant on changes in thermal adaptation (Wells, 1935; Sumner and Wells, 1935; Sumner and Lanham, 1942; Sumner and Doudoroff, 1938). Indeed Sumner and Doudoroff used the change in resistance time to cyanide poisoning as an index of change in thermal adaptation. The change shown in our work appears however to be unique in that the absolute level of respiratory sensitivity is lower at a higher temperature.

While no definite conclusions can be drawn it is interesting to speculate on the change of organization which could bring about this change in respiratory sensitivity. Two phases of the circulatory systems of fish have been shown to be correlated with their respiratory sensitivity. Black (1940) noted that there were significant differences between four species of freshwater fish and the effect of carbon dioxide on the affinity of hemoglobin in whole blood for oxygen, and further, that the fish with the greater sensitivity to carbon dioxide are found in colder habitats. Hart (1945) showed a similar correlation between the stroke output of the heart and the respiratory sensitivity of a series of species. He also pointed out that the influence of the mechanical factor in respiratory transport may at times be so great as to displace the order of respiratory sensitivity of species which might be expected from a comparison of the chemical characteristics of their oxygen transport system. In addition to Hart's material another probable instance of this is found in the marine fish, the tautog (*Tautoga onitis*) and the toadfish (*Opsanus tau*). The respiratory tolerance of these fish is quite different, the toadfish being able to withstand up to 250 mm. of carbon dioxide whereas the tautog can only tolerate 150 mm. (Safford, 1940). The chemical properties of the blood of these fish for transporting oxygen in the presence of carbon dioxide are, however, very similar (Root, Irving, and Black, 1939).

Thus the change in respiratory sensitivity with change in temperature as displayed by goldfish adapted to their thermal environment may be a reflection of changes in either the mechanical or chemical aspects of their circulatory system and the problem remains to see to what extent each may be acting.

There is another change in the organism that may influence the measure of respiratory sensitivity as determined here for the goldfish. The areas enclosed above and to the left of the carbon dioxide-oxygen curves in Figures 1 and 2 are not entirely areas of tolerance in the sense that the animal could remain alive indefinitely under conditions represented by any combination of oxygen and carbon dioxide within these areas. At points in the marginal area near the limiting curve the animal is in a dying condition and its ability to transport oxygen will not depend on its tolerance towards those conditions but upon the length of time its organization can resist the ultimate breakdown. During the part of this period when the ventilatory and circulatory systems are still functioning, sub-minimal amounts of oxygen will be transported to the tissues of the dying animal. While these amounts of oxygen may be sub-minimal from the point of view of maintaining the life of the organism they

may be quite significant in extending the carbon dioxide-oxygen curve considerably beyond the region of tolerance. This effect was well shown by Wiebe *et al.* (1934) who demonstrated that after a fish introduced into a sealed container had extracted oxygen in the presence of a certain pH until it had died, a second subject introduced into the same container could reduce the oxygen still further by a significant amount before it also died.

Thus the decrease in the respiratory sensitivity at higher temperatures as measured by asphyxiation in a sealed bottle could in part be accounted for by increase in resistance of the tissues to oxygen lack or carbon dioxide excess, taking resistance to mean the length of time that an organism can resist a lethal condition before it finally succumbs. However one would not expect this factor to account for anything like the whole of the change in respiratory sensitivity observed.

When conditions are standard with respect to heredity and environment the respiratory sensitivity curves appear to offer a valid physiological yardstick whatever question there may be of exactly what these experiments measure. In the present series of experiments for instance the 20° curve was obtained in 1945 using material of the same age and purchased from the same source as that which was used for the determinations at the other temperatures in 1943, and as will be seen the data are quite consistent.

Necessity for complete acclimation to secure standard results becomes apparent when temperature effect over the biokinetic range at one level of thermal adaptation is considered. We have made two comparisons on fish acclimated to 20° C. In one instance the experiments were conducted in water at 12° C., in the other at 28° C. These data are given in Table II and may be compared with the values given for

TABLE II

*The extent to which goldfish acclimated to 20° C. can reduce the oxygen content of water containing dissolved carbon dioxide at 12° C. and at 28° C. Each pair of values is the mean of four determinations*

Acclimated to 20° Experiments at 12°		Acclimated to 20° Experiments at 28°	
CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg	CO <sub>2</sub> mm. Hg	O <sub>2</sub> mm. Hg
24	5.7	28	7.9
82	5.4	69	7.4
100	8.6	121	11
111	9.8	140	18
127	19	157	60
152	32	169	25
181	100	186	141
		217	184

20° C. in Table I. In both cases the respiratory sensitivity proved to be greater than that measured at the temperature of acclimation. The degree of difference that these results show is somewhat surprising in view of the fact that the difference in temperature at which the experiments were carried out was only 8 degrees in either direction from the acclimation temperature, while at the acclimation level chosen the lower lethal temperature is about 18° C. below and the upper lethal 15° C. above the acclimation temperature.

## SUMMARY

1. By asphyxiating goldfish in sealed bottles of water containing dissolved carbon dioxide and oxygen, it was found that the ability of the fish to use oxygen in the presence of carbon dioxide increases as the acclimation temperature of the fish is raised.

2. Goldfish acclimated at 1° C. are limited in their ability to use oxygen when a tension of 60 mm. or more carbon dioxide is present in the water, whereas goldfish acclimated at 32° C. are not limited in the utilization of oxygen unless the carbon dioxide tension is 200 mm. or more. Fish at intermediate temperatures of 7°, 15°, 20° and 25° C. begin to show a decrease in the ability to use oxygen when they are in the presence of carbon dioxide tensions of 100, 120, 140, and 170 mm. respectively.

3. Reliable results could be obtained only after complete acclimation of the fish to the temperature at which the experiments were made. However, with acclimated fish of similar stock the result could be reproduced from year to year at the same season.

4. The explanation for this change in sensitivity to carbon dioxide in acclimated fish lies possibly in the effects of temperature on both the mechanical transport of the blood and the chemical transport of oxygen by the blood.

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# THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

## THE MARINE BIOLOGICAL LABORATORY

FORTY-NINTH REPORT, FOR THE YEAR 1946—FIFTY-NINTH YEAR

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A. K. PARPART, Princeton University  
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EUGENE F. DUBOIS, Cornell University Medical College  
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H. H. PLOUGH, Amherst College  
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CHARLES PACKARD, *Ex officio*  
L. G. BARTH, to serve until 1947  
WM. RANDOLPH TAYLOR, to serve until 1947  
P. B. ARMSTRONG, to serve until 1948  
P. S. GALTSOFF, to serve until 1948  
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## II. ACT OF INCORPORATION

No. 3170

## COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

*Witness* my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,  
*Secretary of the Commonwealth.*

## III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 10 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be

held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years; and in addition there shall be two groups of Trustees as follows:

#### IV. THE REPORT OF THE TREASURER

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

*Gentlemen:*

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1946.

The accounts have been audited as in recent years by Messrs. Seamans, Stetson, and Tuttle, certified public accountants of Boston. A copy of their report is on file at the Laboratory office and inspection of it by Trustees or other Members of the Corporation will be welcomed.

The principal summaries of their report—The Balance Sheet, Statement of Income and Expense, and Current Surplus Account—are appended hereto as Exhibits A, B, and C.

The financial operations of 1946 may be summarized as follows:

##### *I. Assets*

###### *1. Endowment Assets*

As of December 31, 1946, the total book value of all the Endowment Assets, including the Scholarship Funds, was \$977,199.30, an increase for the year of \$10,427.14 due to profits on securities sold.

These Assets consisted of: marketable securities (bonds, preferred stocks, and common stocks) with a book or cost value of \$889,416.18 and a market value of \$934,042.19; six participations in mortgages on New York City realty costing \$82,491.14, estimated by the Treasurer to have an actual value of \$52,000,000; and \$5,291.98 in uninvested principal cash.

The total current valuation of Endowment Assets was \$991,334.17, \$14,134.87 more than current book value, but \$13,603.44 less than the actual value at the end of the previous year. This slight decline in market values during the year was due chiefly to lower prices for some of the common stocks.

###### *2. Plant Assets*

The total of Plant Assets at the end of the year was \$1,312,963.70, or \$17,884.05 less than at the end of 1945. New expenditures were more than offset by the depreciation charge of \$25,314.41. In addition to heavy expenditures for repairs that were regarded as current expenditures, a total of \$21,425.02 was paid out for improvements to buildings and new equipment that were regarded as capital

items. To take care of some of these payments \$15,000 of the Reserve Fund was used, leaving a balance in the Reserve Fund of \$15,859.89, a substantial portion of which has already been appropriated for repairs and equipment.

### 3. Current Assets

At the end of the year Current Assets had increased by \$7,881.53 to a total of \$220,851.88. Accounts Payable were \$8,411.64. Current Surplus had increased \$6,022.47 to \$202,360.31.

## II. Income and Expenditures

Both income and expense totals were much larger than in recent years. Total Income was \$227,104.26. Total Expense, including \$25,314.41 Reserve for Depreciation, was \$234,537.13, leaving an accounting deficit of \$7,432.87 compared with a surplus of \$9,773.28 for 1945. The 1946 expenditures contained a number of unusual items such as \$11,510.00 for "Special Repairs to the Brick Laboratory" and further "1944 Hurricane Damage Repairs" of \$2,012.50.

On December 31, 1946, current cash on hand amounted to \$28,135.30, but as in the case of the Reserve Fund, a portion of this was designated for further improvements and purchases already authorized.

Noteworthy in the income account are the contributions totalling \$1,070.00 from the Marine Biological Laboratory Associates that were used for purchases of especially needed apparatus and a gift of \$700 from Mrs. W. Murray Crane.

The total receipts of the Supply Department also attained what is believed to be a new high, \$73,394.26. The expenses of the Supply Department, particularly the purchases of specimens from outside sources, also increased however to \$62,527.53, so that the net gain from operations rose only moderately to \$10,866.73.

## EXHIBIT A

### MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1946

#### Assets

#### Endowment Assets and Equities:

Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee .....	\$ 960,062.58
Securities and Cash in Minor Funds .....	17,136.72

\$ 977,199.30

#### Plant Assets:

Land .....	\$ 110,425.38
Buildings .....	1,324,036.67
Equipment .....	193,539.92
Library .....	349,234.22

\$1,977,236.19

Less Reserve for Depreciation ..... 696,981.38

Reserve Fund, Securities and Cash .....	\$1,280,254.81
Book Fund, Securities and Cash .....	15,859.89
	<u>16,849.00</u>

\$1,312,963.70

# REPORT OF THE TREASURER

7

## Current Assets:

Cash .....	\$	28,135.30
Mortgage Note Receivable .....		2,500.00
Accounts Receivable .....		30,114.98

## Inventories:

Supply Department .....	\$	39,083.91
Biological Bulletin .....		18,557.44
		57,641.35

## Investments:

Devil's Lane Property and Accounts Receivable .....	\$	48,055.31
Gansett Property and Accounts Receivable .....		1,074.67
Stock in General Biological Supply House, Inc. ....		12,700.00
Other Investment Securities .....		20,795.00
Retirement Fund Securities and Cash .....		12,757.24
		95,382.22
Prepaid Insurance .....		4,556.59
Items in Suspense (Debits) .....		2,521.44

\$ 220,851.88

Total Assets ..... \$2,511,014.88

## Liabilities

### Endowment Funds:

Endowment Funds .....	\$	958,871.15
Reserve for Amortization of Bond Premiums ..		1,191.43

\$ 960,062.58  
17,136.72

Minor Funds .....

\$ 977,199.30

### Plant Funds:

Mortgage Note Payable .....	\$	5,000.00
Donations and Gifts .....	\$1,172,564.04	
Other Investments in Plant from Gifts and Current Funds .....	135,399.66	1,307,963.70

\$1,312,963.70

### Current Liabilities and Surplus:

Accounts Payable .....	\$	8,411.64
Items in Suspense (Credits) .....	\$	1,751.24
Reserve for Repairs and Replacements .....		8,328.63
Current Surplus .....		202,360.37

\$ 220,851.88

Total Liabilities ..... \$2,511,014.88

## MARINE BIOLOGICAL LABORATORY

## EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE,  
YEAR ENDED DECEMBER 31, 1946

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund .....		\$ 29,966.14		\$ 29,966.14
Library Fund .....		6,000.47		6,000.47
Donations .....		2,170.00		2,170.00
Instruction .....	\$ 11,788.90	9,030.00	\$ 2,758.90	
Research .....	6,329.17	20,645.14		14,315.97
Evening Lectures .....	75.00		75.00	
Biological Bulletin and Membership Dues ..	10,500.17	12,863.15		2,362.98
Supply Department .....	62,527.53	73,394.26		10,866.73
Mess .....	32,567.63	30,069.72	2,497.91	
Dormitories .....	30,640.35	16,880.30	13,760.05	
(Interest and Depreciation charged to above 3 Departments) .....	(26,008.91)			26,008.91
Dividends, General Biological Supply House		17,272.00		17,272.00
Dividends, Other Investment Stocks .....		1,000.00		1,000.00
Rents:				
Bar Neck Property .....	873.71	6,000.00		5,126.29
Janitor House .....	21.45	360.00		338.55
Sale of Library Duplicates, Micro Film, etc.		331.61		331.61
Microscope and Apparatus Rental .....		1,011.47		1,011.47
Sundry Income .....		10.00		10.00
Interest on Mortgage Receivable .....		100.00		100.00
Maintenance of Plant:				
Buildings and Grounds .....	28,337.37		28,337.37	
Apparatus Department .....	8,267.02		8,267.02	
Chemical Department .....	3,859.74		3,859.74	
Library Expense .....	6,664.76		6,664.76	
Workmen's Compensation Insurance ....	435.15		435.15	
Truck Expense .....	157.11		157.11	
Bay Shore Property .....	172.32		172.32	
Great Cedar Swamp .....	22.80		22.80	
General Expenses:				
Administration Expense .....	16,429.92		16,429.92	
Endowment Fund Trustee and Safe- Keeping .....	1,033.70		1,033.70	
Bad Debts .....	352.83		352.83	
Special Repairs 1944 Hurricane Damage ...	2,012.50		2,012.50	
Cost of Living Bonus .....	240.00		240.00	
Gift to Naples Biological Station .....	100.00		100.00	
Special Repairs, Brick Laboratory .....	11,510.00		11,510.00	
Interest .....	312.50		312.50	
Reserve for Depreciation .....	25,314.41		25,314.41	
	\$234,537.13	\$227,104.26	\$124,313.99	\$116,881.12
Excess of Expense over Income carried to Current Surplus .....		7,432.87		7,432.87
		\$234,537.13		\$124,313.99

## EXHIBIT C

MARINE BIOLOGICAL LABORATORY CURRENT SURPLUS ACCOUNT,  
YEAR ENDED DECEMBER 31, 1946

Balance, January 1, 1946 .....		\$196,337.90
Add:		
Transfer from Reserve Fund .....	\$15,000.00	
Reserve for Depreciation Charged to Plant Funds .....	25,314.41	40,314.41
		<u>\$236,652.31</u>
Deduct:		
Excess of Expense over Income for Year .....	\$ 7,432.87	
Payments from Current Funds during Year for Plant Assets:		
Buildings .....	\$ 2,691.13	
Equipment .....	8,324.14	
Library .....	10,409.75	
	<u>\$21,425.02</u>	
Less Received for Plant Assets Sold .....	3,306.90	18,118.12
Transfers to Reserve Fund:		
Portion of Dividends from General Biological Supply House .....	\$ 2,500.00	
Dividends from Crane Co. ....	875.00	
Gansett Property Profits, 1945 .....	464.18	3,839.18
Retirement Fund:		
Payments to Pensioners .....	\$ 3,460.00	
Less Retirement Fund Income .....	200.11	
	<u>\$ 3,259.89</u>	
Loss on Sale of Interest in Real Estate .....	1,030.34	4,290.23
Loss on Sale of Gansett Property in 1946 .....		81.60
Adjustment of Sundry Accounts-Receivable, etc., account of Prior Years .....		529.94
		<u>34,291.94</u>
Balance, December 31, 1946 .....		\$202,360.37

Respectfully submitted,

DONALD M. BRODIE,  
*Treasurer*

## V. REPORT OF THE LIBRARIAN

1946

The sum of \$13,500 appropriated to the library in 1946 was expended as follows: books, \$546.47; serials, \$2,747.01; binding, \$618.39; express, \$74.34; supplies, \$431.05; salaries, \$7,886.13 (\$1,700 of this sum was contributed by the Woods Hole Oceanographic Institution); back sets, \$417.04; insurance, \$45.00; total, \$12,765.43. The cash receipts of the library totalled \$331.61: for microfilms, \$195.03 (\$78.45 expenses paid by the library and accounted above under "sup-

plies"); sale of duplicates, \$135.65; sale of the "Serial List" Biological Bulletin supplement number, \$0.93. This sum, \$331.61, reverts to the laboratory and does not include rent payments for library readers which are collected by the main office. There were 38 library readers accommodated in the library during the summer of 1946, and practically all of these for the whole season, an improvement over 1945 when many of the 49 readers rented desks for short terms.

Of the Carnegie of New York Fund, \$1,534.46 was expended for the completion of four back sets and for the partial completion of one other.

The sum appropriated by the Woods Hole Oceanographic Institution in 1946 for purchases was \$800. A balance of \$802.27 remaining from 1945 made an available total of \$1,602.27. The expenditures amounted to \$1,910.43, overrunning the account by the sum of \$308.16. In addition to the above, the Woods Hole Oceanographic Institution contributed \$1,700 (see above under salaries).

During 1946 the library received 1,065 current journals: 320 (19 new) by subscription to the Marine Biological Laboratory; 43 (15 new) to the Woods Hole Oceanographic Institution; exchanges with the "Biological Bulletin" 433 (13 new; 68 reinstated foreign) and 96 (21 new; 17 reinstated foreign) with the Woods Hole Oceanographic Institution; 165 as gifts to the former and 8 to the latter. The Library acquired 261 books: 81 by purchase of the Marine Biological Laboratory; 53 by purchase of the Woods Hole Oceanographic Institution; 13 gifts by the authors; 22 by the publishers; 48 by miscellaneous donors; 23 from the French Embassy Library, New York City; 1 from Dr. R. S. Lillie; 3 from Dr. Oliver S. Strong; 1 from Dr. Alfred C. Redfield; and 16 from Mrs. Priscilla B. Montgomery. There were 43 back sets of serial publications completed; 15 purchased by the Marine Biological Laboratory (4 with the Carnegie Fund); 7 by the Woods Hole Oceanographic Institution; 5 by exchange of the "Biological Bulletin"; 14 by exchange with the Woods Hole Oceanographic Institution publications; and 2 by gift and exchange of duplicate material. Partially completed sets were 73: purchased by the Marine Biological Laboratory, 10 (1 with the Carnegie Fund); purchased by the Woods Hole Oceanographic Institution, 10; by exchange with the "Biological Bulletin," 9; by exchange with the Woods Hole Oceanographic Institution publications, 11; by gift and exchange of duplicate material, 33.

The reprint additions to the library were 4,618: current of 1945, 841; current of 1946, 97; and of previous dates, 3,680. There were gifts of reprints from Dr. Alfred C. Redfield; Dr. E. G. Butler; and from Dr. L. C. Wyman.

At the end of the year 1946 the library contained 55,312 bound volumes and 142,292 reprints.

It is discouraging to report that no part of the German journals that have been lacking since 1940 have come through. We have heard, however, by letter of February 28, 1947, from the Otto Harrassowitz Company that there is "in preparation" a box of "stored material" that "will leave Berlin in summer via the Library of Congress Mission Berlin." We are hoping to discover whether this means some volumes of our standing order of the war years. If nothing comes to us in this direct way or through other of the Library of Congress "Mission Berlin" shipments I understand that a plan is already under way to reprint such lacking volumes provided a sufficient number of libraries are interested to assure the expense of the undertaking.

## VI. REPORT OF THE DIRECTOR

## TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY

*Gentlemen:*

I submit herewith the report of the fifty-ninth session of the Marine Biological Laboratory for the year 1946.

The past year has seen a continued recovery from war-time conditions. Our attendance increased, especially in the groups of Beginning Investigators and students, in large part due to returning veterans; many foreign journals have begun to come regularly after a lapse of several years; our gross income (and also expense) exceeded that of any recent year; the budget for research equipment was greatly increased; a good start was made in the repair of our buildings; the Lalor Fellowships were established.

1. *Financial*

During the past 10 years notable changes have occurred in the net income from some of our regular sources of revenue. Decreases appear in the return from the Endowments, from Instruction, and from the Mess; increases are seen in Research fees and the Supply Department. Changes in the cost of running the various departments have been due to war conditions. The accompanying table gives only the more important items of the budget. Net incomes are shown, rather than gross incomes. For the Supply Department, the Dormitories, and the Mess, the amounts do not include depreciation and other bookkeeping charges.

## NET INCOME

	Endowments	Dividends	Research	Instruction	Supply Dept.	Dorm.	Mess
1946	35,965	18,270	14,315	(2,760)	14,545	5,290	780
1944	33,010	17,295	3,675	(2,915)	11,780	875	790
1942	36,085	11,420	4,950	(2,065)	1,760	9,275	1,385
1940	44,140	18,940	15,070	(615)	5,030	7,080	2,120
1936	50,190	12,700	10,070	2,130	9,160	6,660	3,125

## EXPENSE

	Bldgs. and Grds.	App. and Chem.	Library	Spec. Repairs	Admin.	Pensions
1946	37,420	15,055	17,075	13,525	16,430	3,460
1944	32,675	6,135	12,315	2,465	15,275	3,460
1942	25,130	9,805	15,335	5,810	12,500	3,460
1940	28,785	19,005	17,780	1,650	12,425	3,710
1936	37,925	17,190	22,140	—	14,910	4,060

Most important is the decline in the return from the Endowments. In 1930 our receipts from these sources amounted to \$57,000. A sharp drop occurred in 1933, and smaller but steady declines each year until 1946. Our income now is less than two-thirds of the 1930 amount. In the meantime, returns from Dividends



have risen, and have made up nearly half of the loss incurred in the Endowment income. The budget for Instruction showed a substantial loss during the war years when attendance was light. With larger numbers of students and an increase in tuition from \$75.00 to \$100.00 the deficit should disappear. The net gain from the Mess has fallen materially. In view of the high cost of food and services we are fortunate to end each season without loss.

On the other hand, the net return from Research fees is now high, due in part to rentals received from the Oceanographic Institution which has occupied rooms throughout the year. The Supply Department has been exceedingly active. For 1946 the gross receipts were over \$73,000, as compared with \$43,000 ten years ago. However, the net income has not increased proportionately, for the cost of specimens purchased from dealers, of containers, chemicals, and other items, has kept down the profits. Salaries have been increased by more than 25 per cent.

The major categories of expense, shown on the lower part of the Table are now, with one exception, only a little above what they were in 1936. During the war we could not obtain many items of scientific equipment and so could not replace outworn apparatus. The budget for the Apparatus Department was, therefore, small. Now when equipment is available we can not buy all that we need because of its high cost. So, although we are spending as much as we did 10 years ago, we obtain far less. Our physical plant fell into disrepair, but is now gradually being put into sound condition. The cost of this work in 1946 is shown in the column Special Repairs.

We stand in need of additional funds to increase our endowments, to improve and enlarge our research facilities, to make further essential repairs in our buildings, to improve the working conditions of the Supply Department, and finally, to replace the wooden laboratories with a modern, well equipped building. The Executive Committee submitted to the Rockefeller Foundation a full statement of our needs with a request for funds to meet all or a part of them. The Foundation, in reply, recognized the importance of these projects but stated that because of its large contribution to the Peking Union Medical College, it could not at this time consider our request. In view of this, the Laboratory, should seek moderate contributions from a number of sources, thus broadening our base of support. One such source is our own membership which has been asked to contribute to a fund for a new collecting boat. The response has been encouraging, the donors expressing their hearty approval of the project.

## *2. Buildings and Grounds*

All of the Laboratory buildings will be in active use during the summer of 1947. The Lecture Hall, which was stripped of its salt water and gas when the Navy occupied it, is now fully restored and will be filled with investigators. Rooms in the Supply Department will also be used for research.

Repairs and replacements have been made elsewhere. The sea wall is now rebuilt, the waterproofing of the Brick Building is finished. The experience of the past winter has proved that the operation was successful. The Dormitory is now undergoing the same treatment. In addition, many of its rooms, left unpainted since the building was erected more than 20 years ago, will be painted. A most desirable improvement is the partial painting of the Auditorium.

Room 106 in the Brick Building has been transformed into an Associates Room. The Trustees, at the Special Meeting in Boston, directed that this be done in response to the request of the Associates who want some place in the Laboratory where they can meet. These friends will constitute an increasingly important part of our membership.

A part of the Devil's Lane Tract is now open, and roads through it have been constructed. Thus far 8 lots have been purchased by members of the Corporation. Now that all lots in the Gansett tract are sold, this new area is the only Laboratory property which is available to investigators who want to solve the housing problem by owning their own homes.

### *3. The Lalor Fellowships*

Through the generosity and cooperation of the Lalor Foundation this Laboratory, during the next five years, can offer to well prepared young investigators, a place to work, adequate equipment, and funds sufficient for their living and traveling expenses. The Fellows will work primarily in the fields of Biophysics, Biochemistry, and Physiological Chemistry, but other fields are not excluded. Dr. Metz who conducted the negotiations with Dr. Burdick of the Lalor Foundation is to be congratulated on their successful conclusion.

### *4. Gifts*

The Laboratory acknowledges with sincere thanks gifts from the following donors:

The Associates: \$1,070 for the purchase of special apparatus.

Mrs. Murray Crane: A \$1,000 bond, the proceeds of which have been used to purchase special apparatus.

Dr. W. D. Curtis: \$100 for carpenter shop equipment.

Dr. A. C. Redfield: \$100 for trees and shrubs.

Mr. Laurence Saunders: \$200 for the bathing beach raft.

### *5. Losses by Death*

The Laboratory has lost by death, since the last meeting of the Trustees, five men, three of whom were closely associated with the institution for more than 50 years.

Prof. William B. Scott, member of the Corporation since 1890; elected Trustee in 1897; Emeritus, 1931.

Prof. Robert A. Harper, Corporation member since 1891; Trustee from 1911 until his retirement in 1926 when he became Emeritus.

Mr. George M. Gray, laboratory assistant in 1891; full time manager of the Supply Department from 1899 until his retirement in 1933.

Prof. Herbert S. Jennings, Trustee from 1905 to 1938, when he was elected Trustee Emeritus.

Prof. Samuel O. Mast, Trustee from 1936 to 1943; Trustee Emeritus until his death.

We honor the memory of these men who through many years greatly helped in the development of this Laboratory.

### 6. Election of Trustees

At the Meeting of the Corporation on August 13, 1946, the following trustees were elected:

#### *Class of 1950*

D. E. S. Brown	A. K. Parpart
D. P. Costello	F. Schrader
M. H. Jacobs	H. B. Steinbach
D. M. Marsland	B. H. Willier

#### *Class of 1949*

F. A. Brown, Jr., to replace W. C. Curtis retired

*Trustee Emeritus*

W. C. Curtis

### 7. There are appended as parts of this report

1. Memorials to Dr. T. H. Morgan and C. E. McClung.
2. The Staff.
3. Investigators and Students.
4. Tabular View of Attendance.
5. Subscribing and Cooperating Institutions.
6. Evening Lectures.
7. Shorter Scientific Papers (Seminars).
8. Members of the Corporation.

Respectfully submitted,

CHARLES PACKARD,  
*Director*

### 1. MEMORIALS TO DECEASED TRUSTEES

*Thomas Hunt Morgan, 1866-1945*

by E. G. Conklin

In the death of T. H. Morgan at Pasadena, California, on December 4, 1945, the world of science lost one of its foremost students of Experimental Zoology and Genetics, and the Marine Biological Laboratory one of its earliest and most eminent members. He was born at Lexington, Kentucky, September 25, 1866, member of a family distinguished in American history for patriotic and military achievements. He graduated from the University of Kentucky in 1886 with the degree of B.S. and was given the M.S. Degree in 1887. In the summer of 1886 he was a student at the marine laboratory at Annisquam, Massachusetts, during the last session of that laboratory, which was the immediate predecessor of the Woods Hole Marine Biological Laboratory. Thus he was the last surviving personal link between the Annisquam and the Woods Hole Laboratories. The Annisquam Laboratory was not widely known, although it was the successor through its founder, Alpheus Hyatt, of

the famous but short-lived laboratory established by Louis Agassiz at Penikese Island in 1872. It would be interesting to learn how Morgan, immediately after his undergraduate course, came to attend this last session of the Annisquam Laboratory, but the fact of his attendance is evidence of his early interest in marine biology.

In the fall of 1886 he entered the Johns Hopkins University as a graduate student in the department of biology taking morphology under W. K. Brooks as his major subject and physiology under H. Newell Martin as his minor. His superior qualities were soon recognized by his appointment as teaching assistant during his first two years and by the University fellowship in biology during his third year. He received the Ph.D. degree in 1890 and was at once appointed to the Adam Bruce post-doctoral fellowship for the following year, part of which he spent on research expeditions in the Bahamas and Jamaica.

On his return from the West Indies in 1891 he was appointed Associate Professor of Biology at Bryn Mawr College, in succession to Professor E. B. Wilson who had been called to Columbia University. Morgan remained at Bryn Mawr as Associate Professor and Professor until 1904, when he was appointed Professor of Experimental Zoology at Columbia, where he remained for 24 years, during which his research work in genetics received world-wide recognition and, together with that of his students and associates, led to many fundamental discoveries regarding the mechanism of heredity and sex. Finally in 1928 he accepted the position of Professor of Biology and Director of the Wm. G. Kerckhoff Laboratories of Biology at the California Institute at Pasadena, where he remained for the balance of his life.

From his first acquaintance with marine biology at Annisquam to the end of his life Morgan was closely associated with marine laboratories, especially during his summer vacations. In 1888 and 1889, while still a graduate student, he occupied the Johns Hopkins Table at the laboratory of the U. S. Fish Commission at Woods Hole. Those were the first two years of the M. B. L. and although he is not listed as a member of this new laboratory for those years, he was closely associated with it from its beginning, for relations between these two Woods Hole laboratories in those days of few workers were very intimate. In 1890 Morgan became an active member of the M. B. L., occupied a room in the laboratory, gave lectures in the Zoology course, and one of the evening lectures, and became a member of the Corporation. In 1899 he became a life member, in 1897 a trustee and in 1936 trustee emeritus. During this long official connection, extending over 55 years, he was always an active collaborator in the plans and work of the laboratory, and was one of its most productive and inspiring investigators. He was for many years a member of the Executive Committee as well as of other special committees and his judgment and cooperation had much to do in shaping policy. Although he believed that once the laboratory was well endowed it should abandon teaching and devote all its resources to research, he nevertheless cooperated in the past and present policy of combining both teaching and research. In his earlier years here he lectured in some of the courses, and throughout his connection with the laboratory he served as a member of the "Staff of Investigation" and supervised the work of many research students. His general lectures to the whole laboratory were many and notable summaries of his research work. Sixteen such "Evening Lectures" are listed in the Annual Reports of the Laboratory and many of these were published in the former series of *Biological Lectures* or in the later *Biological Bulletin*.

He was rarely absent from the sessions of this laboratory, except in 1894, 1895 and 1900 when he was at the Zoological Station in Naples. Even after his chief interests had turned from experiments on marine animals to the genetics of insects he continued to come to Woods Hole summers, transporting his material and continuing his work here. And after his removal to California he and Mrs. Morgan continued to make the long journey to Woods Hole every summer until prevented by war conditions in the past two years. In California he made frequent visits to the marine laboratory of the California Institute at Corona del Mar, which he had established, and his last work there was done last summer on "Normal and Abnormal Development of the Eggs of *Ciona*" and was published about a month after his death.

When he was a student at Johns Hopkins, morphology, which was his chief subject, consisted largely of observational rather than experimental work and was centered to a large extent on the embryology of various classes of animals with the primary purpose of determining their phylogeny and relationships. Morgan's early researches were of this sort. By the time he had taken his Ph.D. he had finished eight papers and in the two following years he published eight more on this general theme. After 1892 he published no papers that were not experimental or analytical. This sudden change in his interests was due in large part to associations formed and interests aroused at Woods Hole and at the Naples Zoological Station. His experiments at Woods Hole began on teleost and echinoderm eggs in 1893, and at Naples in 1894 he collaborated with Hans Driesch on the embryology of ctenophores and began experiments on many other groups. Thereafter he was always interested in the factors controlling development and always had some work going on that subject even when he was more deeply involved in other projects. In all he published about sixty papers and three books on the experimental embryology of animals belonging to ten different phyla or classes. Another field in which he did an enormous amount of work is regeneration, on which he published about forty papers and two books, on some eight classes of animals.

About the time of his removal to Columbia University in 1904 he began work on general cytology and especially on the cytology of sex determination. This led to extensive studies on the Cytological basis of peculiar sex ratios in parthenogenesis and on non-typical Mendelian ratios in general. Here also may be classed his studies on sex-limited and sex-linked heredity together with the causes of secondary sexual characters, as well as his numerous papers on fertility and sterility, which continued intermittently until the end of his life. In this field he published some fifty papers and two books.

Finally in the general field of Mendelism, mutations, and the "constitution of the germ plasm," he published either individually or in collaboration with assistants, about one hundred and twenty-five papers and ten books. Other publications of a more general character, such as those on evolution, natural selection, reviews, and biographies, bring the total number of his publications, as listed in the bound volumes of his reprints in our library and in unbound separates since 1937, to three hundred and seventy separate titles, of which twenty-four are books, some of which are translations into the Dutch, German, Spanish and Russian languages.

This long list of Morgan's published work on a great variety of subjects throws light on many of his personal characteristics. His biological interests were very extensive and he was extraordinarily alert in picking up new leads in research. He worked with great intensity and rapidity and published his journal articles promptly,

and sometimes without adequate caution; as a result he often made mistakes. His laboratory room was always open to callers, but unless their business was important he did not permit them to interrupt his work. He would go right on with what he was doing and would answer questions without looking up from his microscope or dishes. But he was always cordial and helpful and if the business were important he would give it prompt attention. In more leisurely moments he loved to joke and gossip and was gay and full of fun, but in the laboratory he usually kept right on with his work. On one occasion during the first World War I wandered into his room and said, "This War is getting terrible." Without looking up he replied, "What war?" And yet he was as anxious as anyone about the course of events. He dwelt in no "ivory tower," but just then he was most interested in counting flies. I think I have never known any other man who wasted so little time. His work was his life.

He could do good work with the most primitive accommodations and instruments, and he cared little for show and finesse. His work room and table and aquaria might be crowded and apparently disorderly but he usually knew just where to put his hand on what he wanted. His microscope slides and bottles and dishes might be crudely labelled but they served his purpose. In short, he wasted no time on needless techniques but went as directly as possible to the results which he sought.

He disliked organizational and committee work, which is often a great waste of time, but in really important matters he took an active and important part, as in the affairs of the Marine Biological Laboratory, the American support of the Naples Station, the founding of the Journal of Experimental Zoology, and many other institutional and professional matters. In a halting and confused situation no one could be more helpful than he, for with lightening-like rapidity he would isolate the main issues from minor ones and go straight to a logical conclusion. His judgment of men was especially sound and forthright and he did not allow it to be warped by personal sentiments. But he was genuinely kind and affectionate with family, friends, students and associates, and no one learned, except by chance, of his many kindnesses and contributions to persons and institutions. I forbear to say more on this subject out of respect to his well known wishes.

He received abundant recognition and honors from many universities and scientific societies in America and foreign countries; he was president of our leading biological and zoological societies as well as of the more inclusive American Association for the Advancement of Science and the more exclusive National Academy of Sciences; he was a foreign member of the Royal Society and the one and only American zoologist to receive the Nobel Award; but these honors never changed in any respect his sincere and modest behavior.

When he began his epoch-making work on *Drosophila* who could have predicted the amazing discoveries which would be revealed in the course of a few years! It had been customary among students of heredity, following Weismann, to speak of "the architecture of the germ plasm," but practically nothing was known of that architecture except its location in the germ cells. After the work of Morgan and his associates that architecture became a detailed plan with precise location of hereditary factors or genes in chromosome maps, and in turn these maps led to fundamental discoveries in the phenomena of heredity itself. In this work he had the invaluable assistance of students who became his assistants and associates, and his judgment of men was never shown better than in his selection of these associates. Together they made an extraordinary team, working in harmonious cooperation and

in a spirit of great adventure. Morgan was the leader of this team and he received chief recognition and honor for their important discoveries but he always gave full credit to his associates and shared his honors with them. And now this great leader and discoverer is gone from among us but his work and inspiration will remain with us and with future generations.

*Professor C. E. McClung*

*April 5, 1870 to January 17, 1946*

by F. R. Lillie

It is difficult, and indeed impossible in a short space, to do justice to the many-sided life of McClung. Here we record our sorrow and sense of loss to our own institution and express our gratitude for the many services that he rendered us. A graduate in Pharmacology of the University of Kansas in 1892, he received his A.B. Degree there in 1896 and, after study elsewhere, his Ph.D. Degree in 1902. He served as head of the department of Zoology at the University of Kansas until he succeeded the lamented Thomas H. Montgomery at the University of Pennsylvania in 1912. He continued unbroken Montgomery's loyalty and devotion to the M. B. L. and was elected a member of the Corporation and Trustee in 1913. With the exception of the year 1934 when he was visiting professor at Keio University in Tokyo, his membership was continuous until his death.

His first appearance at this Laboratory was as investigator and instructor in Embryology in 1903. Thereafter he was occupied in the work of the University of Kansas until his appointment at the University of Pennsylvania. He was then appointed on the M. B. L. staff of investigations and served throughout his life assisting not only his own students but many others who sought his advice. He also became interested in the library and served as Chairman of the Library Committee from 1923 to 1933 during the great development of the library after the erection of the main building in 1924; he was especially helpful when the General Education Board in 1925 presented \$50,000.00 to be expended over a period of five years for purchase of books, periodicals and pamphlets, and in 1929 \$200,000.00 for endowment of the library.

After the erection of the Crane Laboratory in 1913, the Laboratory rapidly became over-crowded again, and in 1919 the trustees decided that steps should be taken to provide construction and endowment that should be adequate to meet the requirements of a generation of biologists. One of the first steps toward this program was to approach the National Research Council through Dr. McClung, Chairman of the Division of Biology and Agriculture. He was instrumental in the appointment of a committee of his division to report on the situation. Their favorable report was approved by the Executive Board of the Council. This encouraged the presentation of the matter to the Rockefeller Foundation, and the raising of the funds for the great plan was soon well under way. Dr. McClung was also helpful at the laboratory from the start of the new plans. The arrangement of the large lecture room, which has proved so successful, was his suggestion.

Many of us remember Dr. McClung's kindness, simplicity of character and friendliness. These qualities were displayed in many way in the social life of Woods Hole; thus, he served as President of the M. B. L. Tennis Club and also of the

M. B. L. Club, the social club of the students and investigators. Such activities, however, never detracted from his main interests in research, and a significant part of his most famous investigations on chromosomes was carried on here.

He was a member of the National Academy of Sciences, of the American Philosophical Society and of numerous national biological societies, and in most of them occupied positions of trust and influence at various times. He was indeed a consecrated man of science! His memory will long endure.

## 2. THE STAFF, 1946

CHARLES PACKARD, Director, Marine Biological Laboratory, Woods Hole, Massachusetts.

### SENIOR STAFF OF INVESTIGATION

E. G. CONKLIN, Professor of Zoology, Emeritus, Princeton University.

FRANK R. LILLIE, Professor of Embryology, Emeritus, The University of Chicago.

RALPH S. LILLIE, Professor of General Physiology, Emeritus, The University of Chicago.

S. O. MAST, Professor of Zoology, Emeritus, Johns Hopkins University.

A. P. MATHEWS, Professor of Biochemistry, Emeritus, University of Cincinnati.

G. H. PARKER, Professor of Zoology, Emeritus, Harvard University.

## ZOOLOGY

### I. CONSULTANTS

T. H. BISSENETTE, Professor of Biology, Trinity College.

L. L. WOODRUFF, Professor of Protozoology, Yale University.

### II. INSTRUCTORS

F. A. BROWN, Associate Professor of Zoology, Northwestern University, in charge of course.

T. H. BULLOCK, Assistant Professor of Neurology University of Missouri Medical School.

W. D. BURBANCK, Associate Professor of Biology, Drury College.

C. G. GOODCHILD, Professor of Biology, Southwest Missouri State Teachers College.

JOHN H. LOCHHEAD, Instructor in Zoology, University of Vermont.

MADELENE E. PIERCE, Assistant Professor of Zoology, Vassar College.

W. M. REID, Assistant Professor of Biology, Monmouth College.

MARY D. ROGICK, Professor of Biology, College of New Rochelle.

### III. LABORATORY ASSISTANT

ANTOIN BACA, Duke University Medical School.

## EMBRYOLOGY

### I. CONSULTANTS

H. B. GOODRICH, Professor of Biology, Wesleyan University.

### II. INSTRUCTORS

DONALD P. COSTELLO, Professor of Zoology, University of North Carolina, in charge of course.

HOWARD L. HAMILTON, Assistant Professor of Zoology, Iowa State College.



JOHN A. MOORE, Assistant Professor of Zoology, Barnard College.

ALBERT TYLER, Assistant Professor of Embryology, California Institute of Technology.

### III. RESEARCH ASSISTANT

MARJORIE HOPKINS FOX, University of California.

### IV. LABORATORY ASSISTANTS

CATHERINE HENLEY, The Johns Hopkins University.

ELEANOR LERNER, Washington University.

## PHYSIOLOGY

### I. CONSULTANTS

WILLIAM R. AMBERSON, Professor of Physiology, University of Maryland, School of Medicine.

HAROLD C. BRADLEY, Professor of Physiological Chemistry, University of Wisconsin.

WALTER E. GARREY, Professor of Physiology, Vanderbilt University Medical School.

MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania.

### II. INSTRUCTORS

ARTHUR K. PARFART, Associate Professor of Biology, Princeton University, in charge of course.

E. S. GUZMAN BARRON, Associate Professor of Biochemistry, The University of Chicago.

EDWARD CHAMBERS, New York City.

AURIN M. CHASE, Assistant Professor of Biology, Princeton University.

ARTHUR C. GIESE, Associate Professor of Biology, Stanford University.

RUDOLPH T. KEMPTON, Professor of Zoology, Vassar College (absent in 1946).

ROBERT RAMSEY, Associate Professor of Physiology, Medical College of Virginia.

## BOTANY

### I. CONSULTANTS

S. C. BROOKS, Professor of Zoology, University of California.

### II. INSTRUCTORS

MAXWELL S. DOTY, Instructor of Botany, Northwestern University.

HANNAH CROASDALE, Dartmouth College.

## EXPERIMENTAL RADIOLOGY

G. FAILLA, College of Physicians and Surgeons, Columbia University.

L. ROBINSON HYDE, Phillips Exeter Academy, Exeter, N. H.

## LIBRARY

PRISCILLA B. MONTGOMERY (MRS. THOMAS H. MONTGOMERY, JR.), Librarian

DEBORAH LAWRENCE, Assistant Librarian

MRS. ELON JESSUP

MARY A. ROHAN

## APPARATUS DEPARTMENT

E. P. LITTLE, Phillips Exeter Academy, Exeter, N. H., Manager  
J. D. GRAHAM DOROTHY LEFEVRE

## CHEMICAL DEPARTMENT

E. P. LITTLE, Phillips Exeter Academy, Exeter, N. H., Manager

## SUPPLY DEPARTMENT

JAMES MCINNIS, Manager  
D. J. ZINN, Naturalist

RUTH CROWELL GRACE M. WITZELL  
M. B. GRAY W. E. KAHLER F. N. WHITMAN  
A. M. HILTON G. LEHY

## GENERAL OFFICE

F. M. MACNAUGHT, Business Manager  
POLLY L. CROWELL MRS. LILA S. MYERS

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T. E. LARKIN, Superintendent  
W. C. HEMENWAY G. T. NICKELSON, JR.  
R. W. KAHLER T. E. TAWELL  
A. J. PIERCE

## THE GEORGE M. GRAY MUSEUM

GEORGE M. GRAY, Curator Emeritus

## 3. INVESTIGATORS AND STUDENTS

## Independent Investigators, 1946

ABELL, RICHARD G., Assistant Professor of Anatomy, University of Pennsylvania.  
ADDISON, WILLIAM H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.  
ANDERSON, RUBERT S., Assistant Professor of Physiology, University of Maryland.  
ANFENSEN, CHRISTIAN B., Instructor in Biological Chemistry, Harvard Medical School.  
ARMSTRONG, PHILIP B., Professor of Anatomy, College of Medicine, Syracuse University.  
BALL, ERIC G., Associate Professor of Biological Chemistry, Harvard Medical School.  
BARRON, E. S. GUZMAN, Associate Professor of Biochemistry, University of Chicago.  
BARTLETT, JAMES H., Professor of Theoretical Physics, University of Illinois.  
BERGER, CHARLES A., Director, Biological Laboratory, Fordham University.  
BEVELANDER, GERRIT, Associate Professor of Anatomy, New York University.  
BLISS, ALFRED F., Instructor in Physiology and Pharmacology, Albany Medical College.  
BOELL, EDGAR J., Associate Professor of Biology, Yale University.  
BONNER, JOHN T., Junior Fellow, Harvard University.  
BRIDGMAN, JOSEPHINE, Associate Professor of Biology, Limestone College.  
BRINK, FRANK, JR., Research Associate, Johnson Foundation, University of Pennsylvania.  
BROOKS, MATILDA M., Research Associate in Biology, University of California.  
BROOKS, SUMNER C., Professor of Zoology, University of California.  
BROWN, DUGALD E. S., Professor of Physiology, New York University.  
BROWN, FRANK A., JR., Associate Professor of Zoology, Northwestern University.

- BROWNELL, KATHERINE A., Research Associate, Ohio State University.  
BUCK, JOHN, National Institute of Health, Bethesda, Maryland.  
BUDINGTON, ROBERT A., Professor of Zoology, Emeritus, Oberlin College.  
BULLOCK, THEODORE H., Assistant Professor of Anatomy, University of Missouri.  
BURBANCK, WILLIAM D., Professor of Biology, Drury College.  
BURKHOLDER, PAUL R., Professor of Botany, Yale University.  
CANNAN, R. KEITH, Professor of Chemistry, New York University.  
CHAMBERS, ROBERT, Research Professor of Biology, New York University.  
CHASE, AURIN M., Assistant Professor of Biology, Princeton University.  
CHENEY, RALPH HOLT, Chairman of Biology Department, Long Island University.  
CHRYSTALL, FRIEDA L., Teacher of Biology, Julia Richman High School.  
CLAFF, C. LLOYD, Research Fellow in Surgery, Harvard Medical School.  
CLARK, ELEANOR LINTON, Voluntary Research Worker, University of Pennsylvania.  
CLARK, ELIOT R., Professor of Anatomy, University of Pennsylvania School of Medicine.  
CLEMENT, A. C., Associate Professor of Biology, College of Charleston.  
CLOWES, G. H. A., Research Director, Emeritus, Eli Lilly and Company.  
COLE, KENNETH S., Professor of Biophysics, University of Chicago.  
CONKLIN, EDWIN G., Professor of Biology, Emeritus, Princeton University.  
COOPER, KENNETH W., Associate Professor of Biology, Princeton University.  
COPELAND, D. EUGENE, Assistant Professor of Zoology, Brown University.  
COPLEY, ALFRED E., Research Associate, Mount Sinai Hospital.  
COSTELLO, DONALD P., Professor of Zoology, University of North Carolina.  
CROASDALE, HANNAH T., Technical Assistant, Dartmouth College.  
CROUSE, HELEN V., Research Associate in Zoology, University of Pennsylvania.  
CROWELL, SEARS, Associate Professor of Zoology, Miami University.  
CURTIS, W. C., Professor of Zoology, University of Missouri.  
DALCO, ALBERT M., Head of Anatomy and Embryology, Faculty of Medicine, Brussels.  
DALTON, HOWARD C., Instructor in Biology, Brown University.  
DEAN, HELEN W., Department of Anatomy, Harvard Medical School.  
DILLER, IRENE COREY, Research Cytologist, Institute for Cancer Research, Lankenau Hospital.  
DOTY, MAXWELL S., Instructor in Botany, Northwestern University.  
DUMM, MARY E., Instructor in Chemistry, New York University Medical College.  
ELLINGER, TAGE U. H., Professor of Zoology, Howard University.  
ERDMAN, J. GORDON, Fellow, Mellon Institute of Industrial Research.  
FAILLA, G., Professor of Radiology, Columbia University.  
FIGGE, FRANK H. J., Associate Professor of Anatomy, University of Maryland School of Medicine.  
FISKE, VIRGINIA MAYO, Instructor in Zoology, Wellesley College.  
FRISCH, JOHN A., Professor of Biology, Canisius College.  
FROEHLICH, ALFRED, Associate, May Institute for Medical Research.  
FURTH, JACOB, Professor of Pathology, Cornell University Medical College.  
GARREY, WALTER E., Professor of Physiology, Emeritus, Vanderbilt University.  
GIESE, ARTHUR C., Associate Professor of Biology, Stanford University.  
GILBERT, PERRY W., Assistant Professor of Zoology, Cornell University.  
GILMAN, LAUREN C., Assistant Professor of Zoology, University of South Dakota.  
GLASER, OTTO C., Professor of Biology, Amherst College.  
GOODCHILD, CHAUNCEY G., Professor of Biology, Missouri State College.  
GOULD, HARLEY N., Professor of Biology, H. Sophie Newcomb College.  
GRAND, C. G., Research Associate, New York University.  
GRAY, I. E., Professor of Zoology, Duke University.  
GREGG, JOHN R., Instructor in Biology, Johns Hopkins University.  
GRUNDFEST, DR. HARRY, Research Associate, Department of Neurology, Columbia University.  
HARDING, CLIFFORD V., Assistant in Physiology, Brown University.  
HARNLY, MORRIS H., Associate Professor, Washington Square College.  
HARTMAN, FRANK A., Professor and Chairman, Department of Physiology, Ohio State University.  
HARVEY, ETHEL BROWNE, Independent Investigator, Princeton University.  
HARVEY, E. NEWTON, Professor of Physiology, Princeton University.

HAYWOOD, CHARLOTTE, Professor of Physiology, Mount Holyoke College.  
HEILBRUNN, L. V., Professor of Zoology, University of Pennsylvania.  
HIBBARD, HOPE, Professor of Zoology, Oberlin College.  
HOPKINS, HOYT S., Associate Professor of Physiology, New York University.  
HUNTER, FRANCIS ROBERT, Assistant Professor of Zoology, University of Oklahoma.  
HUTCHINGS, LOIS M., Instructor in Biology, New York University.  
IFFT, JOHN D., Assistant Professor of Biology, Simmons College.  
JACOBS, M. H., Professor of General Physiology, University of Pennsylvania.  
JAKUS, MARIE A., Research Associate in Biology, Massachusetts Institute of Technology.  
JENKINS, GEORGE B., Professor of Anatomy, Emeritus, George Washington University.  
JEROME, SISTER FRANCIS, Instructor, Ohio State University.  
JOHLIN, J. M., Associate Professor of Biochemistry, Vanderbilt Medical School.  
KAAN, HELEN W., Associate Professor of Zoology, Wellesley College.  
KEMP, MARGARET, Assistant Professor of Botany, Smith College.  
KEMP, NORMAN E., Instructor in Biology, Wayne University.  
KINDRED, JAMES E., Professor of Anatomy, University of Virginia.  
KLEINHOLZ, LEWIS H., Guggenheim Fellow, Harvard University.  
KLOTZ, JOHN W., Instructor in Biology, Concordia Teachers College.  
KREEZER, GEORGE L., Guggenheim Fellow, Princeton University.  
KRUGELIS, EDITH J., Instructor of Zoology, Vassar College.  
KUNTZ, ELOISE, Assistant in Biology Department, Brown University.  
LAVIN, GEORGE I., In charge of Spectroscopic Laboratory, Rockefeller Institute for Medical Research.  
LAZAROW, ARNOLD, Instructor in Anatomy, Western Reserve Medical School.  
LEFEVRE, PAUL G., Instructor in Physiology, University of Vermont, College of Medicine.  
LILLIE, RALPH S., Professor of Physiology, Emeritus, The University of Chicago.  
LILLY, DANIEL M., Assistant Professor in Biology, Providence College.  
LLANA, DR. ALFRED H., Assistant Director, University of Chile.  
LOCHHEAD, JOHN H., Assistant Professor of Zoology, University of Vermont.  
LUCKE, BALDUIN, Professor of Pathology, University of Pennsylvania School of Medicine.  
LYNCH, REV. WM. F., Teaching Fellow, New York University.  
MANGINELLI, PIERO, Assistant, Faculdade de Medicina, Sao Paulo, Brazil.  
MARINELLI, LEONIDES D., Physicist, Memorial Hospital.  
MARMONT, GEORGE H., Assistant Professor of Biophysics, University of Chicago.  
MARSLAND, DOUGLAS, Associate Professor of Biology, New York University.  
MAST, S. O., Professor of Zoology, Emeritus, Johns Hopkins University.  
MATHEWS, ALBERT P., Professor of Biochemistry, Emeritus, University of Cincinnati.  
MENDES, ERASMO GARCIA, Assistant Professor, University of Sao Paulo, Brazil.  
MENKIN, VALY, Assistant Professor of Pathology, Duke University School of Medicine.  
METZ, CHARLES W., Director, Zoological Laboratory, University of Pennsylvania.  
MOORE, JOHN A., Assistant Professor of Zoology, Barnard College.  
NACHMANSOHN, DAVID, Research Associate in Neurology, Columbia University.  
NELSON, LEONARD, Independent Investigator, University of Pennsylvania.  
NORTROP, JOHN H., Member of the Institute, Rockefeller Institute for Medical Research.  
OPPENHEIMER, JANE M., Assistant Professor of Biology, Bryn Mawr College.  
ORMSBEE, RICHARD A., Associate, Sloan-Kettering Institute, Memorial Hospital.  
ORR, PAUL R., Assistant Professor, Brooklyn College.  
OSTER, ROBERT H., Professor of Physiology, University of Maryland School of Medicine.  
OSTERHOFF, W. J. V., Member Emeritus, Rockefeller Institute for Medical Research.  
PALADE, GEORGE, Assistant Department of Anatomy, University of Bucharest, Rumania.  
PARPART, ARTHUR K., Professor of Biology, Princeton University.  
PIERCE, MADELENE E., Associate Professor of Zoology, Vassar College.  
PIERSON, BERNICE F., Independent Investigator, Johns Hopkins University.  
PLOUGH, HAROLD H., Professor of Biology, Amherst College.  
POULSON, D. F., Assistant Professor of Biology, Yale University.  
RAKESTRAW, NORRIS W., Research Associate, Woods Hole Oceanographic Institute.  
RAMSEY, ROBERT W., Associate Professor of Physiology, Medical College of Virginia.  
RANKIN, JOHN S., Assistant Professor of Zoology, University of Connecticut.

- REID, W. MALCOLM, Head of Biology Department, Monmouth College.  
 REINHARD, EDWARD G., Professor of Biology and Head of Department, Catholic University of America.  
 RIS, HANS, Assistant in Physiology, Rockefeller Institute.  
 ROBBIE, WILBUR A., Research Associate, State University of Iowa.  
 ROGICK, MARY DORA, Professor of Biology, College of New Rochelle.  
 RYAN, FRANCIS, Assistant Professor of Zoology, Columbia University.  
 SCHAEFFER, A. A., Chairman Department of Biology, Temple University.  
 SCHARRER, ERNST, Associate Professor of Anatomy, University of Colorado School of Medicine.  
 SCHMIDT, GERHARD, Senior Research Fellow in Medicine, Tufts College Medical School.  
 SCHNEYER, LEON H., Instructor, New York University Dental College.  
 SCOTT, SISTER FLORENCE MARIE, Professor of Biology, Seton Hill College.  
 SHANES, ABRAHAM M., Assistant Professor of Physiology, New York University College of Dentistry.  
 SHERESHEFSKY, J. LEON, Professor of Physical Chemistry, Howard University.  
 SICHEL, DR. F., Professor of Physiology, University of Vermont College of Medicine.  
 SLIFER, ELEANOR H., Assistant Professor of Zoology, State University of Iowa.  
 STEINBACH, H. B., Associate Professor of Zoology, Washington University.  
 STEWART, DOROTHY R., Research Associate in Physiology, University of Pennsylvania Medical School.  
 STOKEY, ALMA G., Professor of Botany, Emeritus, Mount Holyoke College.  
 STUNKARD, HORACE H., Professor of Biology, New York University.  
 STURTEVANT, ALFRED H., Professor of Genetics, California Institute of Technology.  
 TAFT, CHARLES H., Associate Professor of Pharmacology, University of Texas.  
 TAYLOR, WM. RANDOLPH, Professor of Botany, University of Michigan.  
 TEWINKEL, LOIS E., Associate Professor of Zoology, Smith College.  
 THOMPSON, BETTY F., Instructor in Botany, Connecticut College for Women.  
 TODD, EDGAR W., Visiting Investigator, Rockefeller Institute.  
 TRACY, HENRY C., Professor of Anatomy, University of Kansas.  
 TYLER, ALBERT, Assistant Professor of Embryology, California Institute of Technology.  
 VILLE, CLAUDE A., Instructor in Biological Chemistry, Harvard Medical School.  
 WAINIO, WALTER W., Assistant Professor, New York University College of Dentistry.  
 WARNER, ROBERT C., Assistant Professor of Chemistry, New York University College of Medicine.  
 WEBER, NEAL A., Associate Professor of Anatomy, University of North Dakota.  
 WEISS, DR. PAUL, Professor of Zoology, University of Chicago.  
 WENRICH, D. H., Professor of Zoology, University of Pennsylvania.  
 WHITING, ANNA R., Instructor in Zoology, University of Pennsylvania.  
 WHITING, P. W., Associate Professor of Zoology, University of Pennsylvania.  
 WICHTERMAN, RALPH, Assistant Professor of Biology, Temple University.  
 WIERCINSKI, FLOYD J., Chairman and Professor of Biology, Lewis College of Science and Technology.  
 WILBER, CHARLES G., Instructor in Physiology, Fordham University.  
 WILLIER, B. H., Professor of Zoology, Johns Hopkins University.  
 WOODWARD, ARTHUR A., JR., Research Assistant in Zoology, University of Pennsylvania.  
 WRINCH, DOROTHY, Lecturer, Smith College.  
 YNTEMA, CHESTER L., Assistant Professor of Anatomy, Cornell University Medical College.

#### Beginning Investigators, 1946

- BENDER, ALBERT, Investigator, University of Pennsylvania.  
 BENEDICT, DORA, Student, Harvard Medical School.  
 BERG, GEORGE G., Graduate Student, Columbia University.  
 BLUMENTHAL, GERTRUDE, University of Pennsylvania.  
 BRUST, MANFRED, Teaching Fellow in Biology, Washington Square College.  
 CHAMBERS, EDWARD L., Research Associate, University of California.  
 DOUGLIS, MARJORIE B., Assistant in Zoology Department, University of Chicago.  
 FISK, ALBERT A., Brown Fellow in Pathology, Yale University School of Medicine.

GOREAU, THOMAS F., Student, University of Pennsylvania School of Medicine.  
GOULD, DAVID, Voluntary Investigator, New York University College of Medicine.  
GREEN, JAMES W., Graduate Student, Princeton University.  
GREENBERG, RUVEN, Graduate Student, Ohio State University.  
GREGORY, JOHN C., Graduate Student, Yale University.  
KOZAM, GEORGE, Graduate Student and Research Assistant, New York University Graduate School.  
LEIN, JOSEPH, Graduate Student, Princeton University.  
LOVELACE, ROBERTA, Teaching Fellow, University of North Carolina.  
MCLEAN, DOROTHY J., Demonstrator, University of Toronto.  
MOUL, EDWIN T., Teaching Fellow, University of Pennsylvania.  
NARAHARA, HIROMICHI, Columbia College of Physicians and Surgeons.  
RECKNAGEL, RICHARD O., Graduate Student, University of Pennsylvania.  
REMOND, A., Research Fellow, Johnson Foundation, University of Pennsylvania.  
ROGER, HENRY C., Student, Deerfield Academy.  
ROYS, CHESTER C., University of Chicago.  
SHENG, T. C., Graduate Student, Columbia University.  
TAYLOR, BABETTE, Graduate Student, Washington University.  
WEINER, MILTON H., Student, Western Reserve University.  
WEISS, MICHAEL S., Teaching Fellow in Biology, New York University.  
WHITE, ELIZABETH L., Graduate Student, Bryn Mawr College.  
WILSON, T. HASTINGS, University of Pennsylvania Medical School.  
WILSON, WALTER L., Graduate Student, University of Pennsylvania.

#### Research Assistants, 1946

ABRAMSKY, TESS A., Research Assistant, Rockefeller Institute for Medical Research.  
BERMAN, MARJORIE, Research Assistant, Columbia University.  
BURT, AGNES S., Research Assistant, University of Chicago.  
CAMPBELL, MARGARET L., Research Assistant, California Institute of Technology.  
CARLSON, FRANCIS D., Research Assistant, Johnson Foundation, University of Pennsylvania.  
DEFALCO, ROSE H., Research Assistant, University of Pennsylvania.  
DEY, THOMAS E., Research Assistant, Princeton University.  
DIETZ, ALMA, Research Assistant, American International College.  
FOX, MARJORIE H., Research Fellow, University of California.  
HOPKINS, C. NADINE, Graduate Student, Northwestern University.  
KERSCHNER, JEAN, Research Assistant, University of Pennsylvania.  
LERNER, ELEANOR, Fellow in Zoology, Washington University.  
MITCHELL, CONSTANCE, Research Assistant, University of Pennsylvania.  
MITCHELL, ROBERT F., Research Assistant, New York University.  
NORRIS, KARL H., Electronic Engineer, University of Chicago.  
RALL, WILFRID, Graduate Fellow in Biophysics, University of Chicago.  
ROBBIE, JEAN, Technical Assistant, Ohio State University.  
ROSEN, MRS. DAVID, Research Assistant, Yale University.  
ROTHERBERG, MORTIMER A., Research Assistant, College of Physicians and Surgeons, New York University.  
SERIN, B. GOLDIS, Research Assistant, University of Pennsylvania.  
ULLED, ELIAS, Research Assistant, Temple University Medical School.  
VISENIAC, WOLF, Research Assistant, Washington University.  
WHEELER, CHARLES B., Part time Instructor, University of Kansas.  
WILLIS, MARIAN, Research Assistant, University of Pennsylvania.  
ZIEGLER, BERNICE, Laboratory Assistant, Washington University.

#### Library Readers, 1946

AMBERSON, WILLIAM R., Professor of Physiology, University of Maryland School of Medicine.  
BAKER, GLADYS E., Associate Professor of Plant Science, Vassar College.  
BENNIGHOF, C. L., Assistant Professor, Western Maryland College.

- BLOCH, ROBERT, Research Associate in Botany, Yale University.  
 BLUM, HAROLD F., Guggenheim Fellow.  
 BOCHER, CAROL A., Research Assistant, The Johns Hopkins University.  
 CASSIDY, HAROLD G., Assistant Professor of Chemistry, Yale University.  
 COX, EDWARD H., Professor of Chemistry, Swarthmore College.  
 DISCHE, ZACHARIAS, Visiting Scholar, College of Physicians and Surgeons, New York University.  
 FRIEDEMANN, ULRICH, Head of Department of Bacteriology, Jewish Hospital, Brooklyn.  
 FRIES, E. F. B., Assistant Professor of Biology, City College of New York.  
 GATES, R. R., Professor of Botany Emeritus, University of London.  
 GRANT, MADELINE P., Member Science Faculty, Sarah Lawrence College.  
 GUDERNATSCH, FREDERICK, Visiting Professor, New York University.  
 GUREWICH, VLADIMIR, Assistant Visiting Physician, Bellevue Hospital.  
 HAMILTON, HOWARD L., Assistant Professor of Zoology, Iowa State College.  
 KELLER, RUDOLPH, Researcher, The Robinson Foundation, Inc.  
 KRASNOW, FRANCES, Head of Department of Research, Guggenheim Dental Foundation.  
 LOEWI, OTTO, Research Professor of Pharmacology, New York University College of Medicine.  
 LOW, EVA M., Graduate Student, Yale University.  
 LOWENSTEIN, OTTO, Clinical Professor of Neurology, New York University College of Medicine.  
 McDONALD, SISTER ELIZABETH SETON, Professor of Biology, College of Mt. Saint Joseph-on-the-Ohio.  
 MAVOR, JAMES WATT, Research Professor of Biology, Union College.  
 MEYERHOF, OTTO, Research Professor of Biochemistry, University of Pennsylvania.  
 MORRIS, SAMUEL  
 MOSCHCOWITZ, ELI, Assistant Professor Chemical Medicine, Columbia University.  
 PONDER, ERIC, Research Investigator, The Nassau Hospital.  
 PRICE, WINSTON H., Research Assistant, Washington University Medical School.  
 SANDOW, ALEXANDER, Assistant Professor of Biology, New York University, Washington Square College.  
 SAVAGE, LEONARD J., Special Rockefeller Fellow, University of Chicago.  
 SCHERANK, A. R., Assistant Professor of Physiology, University of Texas.  
 SHWARTZMAN, GREGORY, Head of Department of Bacteriology, Mt. Sinai Hospital.  
 SPEIDEL, CARL C., Professor of Anatomy, University of Virginia.  
 STERN, KURT G., Adjunct Professor of Biochemistry, Polytechnic Institute of Brooklyn.  
 STRAUS, WILLIAM L., JR., Associate Professor of Anatomy, Johns Hopkins University.  
 WHITE, MICHAEL J. D., Reader in Zoology, University College, London.  
 WINSOR, CHARLES P., Assistant Professor of Biology, Johns Hopkins School of Hygiene.  
 ZORZOLI, ANITA, Instructor in Physiology, Washington University School of Dentistry.

### Students, 1946

#### BOTANY

- CLIFFORD, SISTER ADELE, S. C., Fordham University.  
 FAHEY, ELIZABETH MARY, Student, Boston University.  
 FRAAS, JANET LOIS, Student, Wheaton College.  
 GARNIC, JUSTINE, Student, Carnegie Institute of Technology.  
 KEEFFE, MARY M., Student, Fordham University.  
 TORREY, ANNA MARSH, Student, Swarthmore College.  
 URQUHART, BETTY A., Instructor in Botany, Wheaton College, Wheaton, Illinois.  
 VISHNIAC, WOLF, Research Assistant, Washington University.  
 WILSON, MARIE ELLEN, Student, Western Maryland College.

#### EMBRYOLOGY

- BANNER, ALBERT H., Assistant Professor, University of Hawaii.  
 BARISH, NATALIE, Student, Goucher College.

BRINSLEY, BERTRAM, Graduate Student, New York University.  
 BRUCH, PAUL ROBERT, Student, Wesleyan University.  
 BUCKLIN, DONALD HARTWELL, 124 Woodbine Street, Providence, Rhode Island.  
 DiDEA, ARTHUR, Assistant in Zoology, Washington University.  
 EBERT, JAMES DAVID, Student, Johns Hopkins University.  
 EISENBERG, NORMA, Graduate Student, Brooklyn College.  
 FITCH, NAOMI SELMA, Student, McGill University.  
 FLOOD, REV. FRANCIS X., S. J., Assistant Professor of Biology, Canisius College.  
 FRIEDMAN, FLORENCE L., Brooklyn College.  
 GALBREATH, JEAN L., Student Assistant, Elmira College.  
 HARRISON, JOHN W., Graduate Student, Washington and Jefferson College.  
 JONES, ALBERTA S., Student, Howard University.  
 KUFF, EDWARD LOUIS, Student, Johns Hopkins Medical School.  
 LEIGH, WALTER HENRY, Instructor, Chicago City Junior College.  
 LIU, CHIEN-KANG, McGill University.  
 MARKERT, CLEMENT LAWRENCE, Graduate Assistant, Johns Hopkins University.  
 MOORE, ELLEN LOUISE, Student, Wellesley College.  
 MORRIS, DAVID MARKLAND, JR., Graduate Assistant and Student, Indiana University.  
 MULLALLY, REV. WALTER BERNARD, Instructor in Biology, St. Anselm's College.  
 MURLAND, RICHARD LEE, Graduate Student, Washington and Jefferson College.  
 PEQUEGNAT, WILLIS EUGENE, Assistant Professor of Zoology, Pomona College.  
 SCHECTER, DAVID EDWARD, McGill University.  
 SEAMAN, ARLENE R., Instructor in Zoology, Cornell University.  
 SHAPIRO, ESTHER MAY, Student, Goucher College.  
 TATUM, ANNE, Student, Rosemont College.  
 TIEMEIER, OTTO W., Graduate Fellow, University of Illinois.  
 TUTTLE, RUTH FRANCES, Instructor, Carnegie Institute of Technology.  
 UBER, VIRGINIA MAE, Assistant in Biology, Pennsylvania College for Women.

### PHYSIOLOGY

ABAJIAN, JOHN, JR., Associate Professor of Anaesthesiology, University of Vermont.  
 BENEDICT, DORA, Student, Harvard Medical School.  
 BERG, GEORGE G., Graduate Student, Columbia University.  
 CLARK, CARL CYRUS, Assistant, Columbia University.  
 CONNELLY, CLARENCE M., Graduate Student, Cancer Fellow, Johnson Foundation. University of Pennsylvania.  
 CRAPSTER, WIRT PATTERSON, Student, Taneytown, Maryland.  
 GREGG, JAMES H. Graduate Student, University of Miami.  
 GREGORY, JOHN C., Graduate Student, Yale University.  
 GUTHE, KARL FREDERICK, Graduate Student, Harvard University.  
 HARTING, JANEY, Undergraduate Assistant, Washington University.  
 HUNTER, FRANCIS ROBERT, Assistant Professor, University of Oklahoma.  
 JOFTES, DAVID LION, Graduate Student, Tufts College.  
 JOHNSON, SHIRLEY ALMA, Research Assistant, University of Toronto.  
 KEMP, NORMAN EVERETT, Instructor in Biology, Wayne University.  
 LEVIN, ILENE B., Goucher College.  
 MARSHALL, JEAN McELROY, Instructor in Physiology, Mount Holyoke College.  
 McPHEE, GWELDA S., Assistant in Physiology, Vassar College.  
 MENDES, ERASMO GARCIA, Assistant Professor, University of Sao Paulo, Brazil.  
 NARAHARA, HIROMICHI TSUDA, Columbia University.  
 NELSON, THOMAS CLIFFORD, Graduate Student, Assistant, Columbia University.  
 ROYS, CHESTER C., University of Chicago.  
 SASLOW, HERBERT B., Laboratory Instructor and Graduate Student, University of Illinois.  
 SHULMAN, NAHUM RAFAEL, Student, Johns Hopkins Medical School.  
 SILBER, EARLE, Student, University of Maryland Medical School.  
 ST. GEORGE, ROBERT C. C., JR., Graduate Student, Harvard University.  
 UMBARGER, H. EDWIN, 239 West Fifth Street, Mansfield, Ohio,



## ZOOLOGY

- AMBERSON, MARGARET MARY, Oberlin College.  
BANNER, ALBERT HENRY, Assistant Professor of Zoology, University of Hawaii.  
BERGQUIST, JEANNE BODIN, Barnard College.  
BERNSAW, MARGARET IDA, Wilson College.  
BINGHAM, BARBARA ALICE, University of Michigan.  
CANDELAS, GUSTAVO, Assistant Instructor, University of Puerto Rico.  
CATTELL, ELLY, Student, Cornell University.  
CHADWICK, JOHN B., Harvard University.  
CHIVERS, MIRIAN E., Howard University.  
DENTON, WINSLOW CROCKER, Graduate assistant, Cornell University.  
EDWARDS, JOHN PARMAN, Student, Drury College.  
EHRENTHEIL, SUSANNE JUDITH, Radcliffe College.  
EMERSON, JULIA RIPLEY, Student, Wellesley College.  
ENDERS, ABBIE GERTRUDE, Student, Swarthmore College.  
FELD, EMILY ANN, Student, University of North Carolina.  
FERGUSON, EDWARD L., Student, Wesleyan University.  
FOLEY, JOSEPH BRENDAN, Graduate student, Yale University.  
FOREMAN, DAREL LOIS, Student Assistant, George Washington University.  
FULLERTON, ANN ELIZABETH, Student, Western Maryland College.  
GEHR, AGNES ROBBINS, Student, Western Reserve University.  
GESE, EDWARD CHARLES, Graduate Assistant, New York University.  
GESE, PHYLLIS KRING, Student, New York University.  
HACKETT, THOMAS PAUL, 573 Purcell Avenue, Cincinnati, Ohio.  
HAND, CADET HAMMOND, JR., Student, University of Connecticut.  
HOPKINS, AMOS L., JR., Student, Harvard College.  
HOFF, WILLIAM BEECHER, Graduate student, Purdue University.  
HUMPHREY, JUDITH M., Student, University of Connecticut.  
JAKOWSKA, SOPHIE, Fordham University.  
KENYON, V. PATRICIA, Graduate assistant, Brown University.  
KRAMER, DAVID, Clark University.  
LIBERTY, ALFRED V., Graduate student, Fordham University.  
LIU CHIEN-KANG, Graduate student, McGill University.  
MACHLIS, GERTRUDE RAFFERTY, Assistant in Zoology, University of Illinois.  
MEINKOTH, NORMAN AUGUST, Assistant in Zoology, University of Illinois.  
MENDES, MARTA VANNUCCI, Assistant Professor, University of Sao Paulo.  
MILLER, MARY ELIZABETH, Student, Western Maryland College.  
MORRIS, DAVID MARKLAND, JR., Graduate assistant in Zoology, Indiana University.  
MOULTON, JAMES MALCOLM, Undergraduate Laboratory assistant, Massachusetts State College.  
PETERS, REV. W., Graduate student, Yale University.  
POLLENS, NORMAN BURTON, Assistant in Zoology, University of Rochester.  
RICE, MARY ESTHER, Drew University.  
ROBINSON, EDWIN JAMES, JR., Graduate assistant, New York University.  
SANDERSON, MARGARET CRASSONS, Vassar College.  
SASLOW, HERBERT B., Graduate assistant, University of Illinois.  
SEITNER, PHILIP G., Graduate Assistant, Purdue University.  
SMITH, THOMAS C., Student, Oberlin College.  
SULLIVAN, REV. THOMAS DONALD, Fordham University.  
SWANSON, ANN TERESA, Graduate Assistant, Tufts College.  
THOMPSON, MARY JANE, Graduate student, Yale University.  
VANHOESSEN, DRUSILLA, Student, University of Pennsylvania.  
VIVIAN, JANET, Graduate student, Radcliffe College.  
WARNER, KATHLEEN LOUISE, Instructor, Mundelein College.  
WARTERS, MARY ELLEN, Student, Oberlin College.  
WETMORE, KATHERINE BERYL, Student, Radcliffe College.  
WILLIAMS, ERNEST E., Columbia University.

## 4. TABULAR VIEW OF ATTENDANCE

	1942	1943	1944	1945	1946
INVESTIGATORS—Total .....	201	160	193	212	267
Independent .....	132	89	112	138	175
Under instruction .....	16	19	11	10	29
Library readers .....	28	35	50	38	38
Research assistants .....	25	17	20	26	25
STUDENTS—Total .....	74	68	75	96	122
Zoology .....	36	47	37	55	57
Embryology .....	24	13	23	23	30
Physiology .....	6	8	10	13	26
Botany .....	8	—	5	5	9
TOTAL ATTENDANCE .....	275	228	276	308	389
Less persons registered as both students and investigators	2	6	1		
	273	222	275		
INSTITUTIONS REPRESENTED—Total .....	126	116	106	124	141
By investigators .....	83	70	74	100	102
By students .....	43	41	41	49	56
SCHOOLS AND ACADEMIES REPRESENTED .....					
By investigators .....	2	2	1	2	2
By students .....	—	1	2	2	—
FOREIGN INSTITUTIONS REPRESENTED .....					
By investigators .....	—	2	2	1	7
By students .....	—	—	3	—	5

## 5. SUBSCRIBING AND COOPERATING INSTITUTIONS

1946

## Cooperating Institutions

Amherst College	Ohio State University
Barnard College	Princeton University
Bryn Mawr College	Rockefeller Institute for Medical Research
The Catholic University of America	State University of Iowa
Columbia University	Syracuse University Medical School
Cornell University	Temple University
Cornell University Medical College	Tufts College
Duke University	University of Chicago
Fish and Wild Life Service, U. S.	University of Cincinnati
Department of the Interior	University of Illinois
Fordham University	University of Maryland Medical School
Goucher College	University of Missouri
Harvard University	University of Pennsylvania
Harvard University Medical School	University of Pennsylvania School of Medicine
Johns Hopkins University	University of Rochester
Lankenau Hospital Research Institute	Vassar College
Eli Lilly and Company	Washington University
Massachusetts Institute of Technology	Wellesley College
Mount Holyoke College	Wesleyan University
New York University	Western Reserve Medical School
New York University College of Medicine	Wheaton College
New York University School of Dentistry	Wilson College
New York University, Washington Square	Woods Hole Oceanographic Institute
College	Yale University
Oberlin College	

## Subscribing Institutions

American Philosophical Society (Penrose Fund)	Macy Foundation
Biological Institute, Philadelphia, Pennsylvania	Memorial Hospital
Brown University	Miami University
California Institute of Technology	Pomona College
Canisius College	Radcliffe College
Carnegie Institute of Technology	St. Anselm's College
College of Mount Joseph-on-the-Ohio	St. Michael's College
Connecticut College for Women	University of Kansas
Elmira College	University of Texas
Johns Hopkins Medical School	University of Toronto
Lewis College of Science and Technology	University of Vermont
	Vanderbilt University Medical School
	Western Maryland College

## 6. FRIDAY EVENING LECTURES, 1946

Friday, June 28

DR. JANE OPPENHEIMER ..... "The Organization of the Teleost Blastoderm."

Friday, July 5

PROF. E. N. HARVEY ..... "The Effects of High Velocity Missiles on Tissue."

Friday, July 12

DR. BENJAMIN W. ZWEIFACH ..... "The Relation of Metabolic Derangements of Liver and Kidney to Peripheral Vascular Reactions."

Friday, July 19

DR. E. W. DEMPSEY ..... "Observations on the Chemical Cytology of Several Mammalian Tissues."

Friday, July 26

PROF. K. S. COLE ..... "A Bomb and Biology."

Friday, August 2

DR. E. J. COHN ..... "The Separation of Plasma into Fractions Varying in Chemical and Biological Properties."

Friday, August 9

DR. ERIC G. BALL ..... "Biochemical Studies on the Malarial Parasite."

Friday, August 16

PROF. M. H. JACOBS ..... "Cell Membranes, Cell Permeability and Cell Volume."

Friday, August 23

DR. E. J. BOELL ..... "Biochemical Differentiation during Amphibian Development."

## OTHER LECTURES

Thursday, July 18

Kodachrome Motion Picture

C. LLOYD CLAFF ..... "Cardiac Resuscitation—Resuscitation Method after Asphyxia. Heart Defibrillation to Control Ventricular Fibrillation during Heart Surgery."

Thursday, July 25

DR. HOWARD MEYERHOF ..... "The Crisis in Science Legislation."

Thursday, August 15

PROF. HARLOW SHAPLEY ..... "Internationalism in Science."

Wednesday, August 21

PROF. J. H. BARTLETT ..... "A Possible Mechanism of Nerve Action."

Saturday, August 31

DR. PAUL S. GALTSOFF ..... "The Atom Bomb Test at Bikini."

## 7. SEMINARS, 1946

Tuesday, July 9

DR ALBERT TYLER ..... "Inhibition of Fertilization in Sea Urchins by means of Univalent Antibodies vs. Antifertilizin."

DR. W. A. ROBBIE ..... "The Cyanide Sensitivity of the Unfertilized Sea Urchin Egg."

DR. J. E. KINDRED ..... "The Effects of Mustard Vesicants on the Hemopoietic Organs."

Tuesday, July 16

DR. A. F. BLISS ..... "Intermediate Steps in the Visual Cycle."

DR. A. M. SHANES ..... "The Role of Metabolism in the Injury Potential of Frog Nerve."

DR. W. H. PRICE ..... "Adenosine Triphosphatase, Myosine, Actin, and their Relation to the Mechanico-Chemical Coupling of Muscle."

Tuesday, July 23

M. M. BROOKS ..... "Oxidation-reduction Studies as a Clue to the Mechanism of Fertilization of Marine Eggs."

C. L. YNTEMA ..... "An Analysis of Induction of the Auditory Vesicle in the Salamander."

J T. BONNER ..... "Aspects of Morphogenesis in the Slime Molds."

Tuesday, July 30

DR. ERNST SCHARER ..... "Chemical Sense and Taste in the Sea Robin, *Prionotus*."

DR. CLAUDE A. VILLEE ..... "Studies of the Respiration of the Imaginal Discs of *Drosophila*, using the Cartesian Diver Ultramicrorespirometer."

DR. C. B. ANFINSEN AND ..... "The Action of Naphthaquinone Antimalarials on Respiratory Enzymes."

DR. E. G. BALL

Tuesday, August 6

DR. A. R. WHITING

DR. H. C. GEORGE ..... "X-Ray Induced Visible and Dominant Lethal Mutations in *Habrobracon* Eggs."

DR. P. B. ARMSTRONG ..... "Specificity of Cholin Esterase."

DR. T. H. BULLOCK ..... "The Giant Synapse in the Stellate Ganglion of the Squid as a Physiological Preparation."

DR. BERTA SCHARRER ..... "The Endocrine Function of the Corpora allata in Insects."

Tuesday, August 13

DR. KATHARINE A. BROWNELL ..... "Evidence of a New Factor from the Adrenal."

DR. F. A. HARTMAN ..... "Hyperactivity of the Adrenal Cortex."

DR. ARNOLD LAZAROW ..... "Studies on the Mechanism of Production of Diabetes with Alloxan."

DR. DOROTHY WRINCH ..... "On the Nature of Biological Specificity."

Tuesday, August 20

DR. C. G. WILBER ..... "A Comparative Study of the Lipids in some Marine Annelids."

DR. C. A. BERGER ..... "Polyploid Mitosis as a Normal Factor in the Development of *Allium cepa*."

DR. P. W. WHITING ..... "Reproductive Economy in Close-crossed Species with Haploid Males."

DR. R. G. SCHMIEDER ..... "Hippocrates on Semen and on the Origin of the Child."

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THE PERIOD OF QUIESCENCE IN THE RESPONSE TO LIGHT  
BY AMOEBA  
(THE RESPONSE TO LIGHT BY AMOEBA)

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INTRODUCTION

As is well known, an amoeba, when subjected to sudden illumination, responds by cessation of movement, and, as this cessation does not take place immediately, an interval exists between the increase in illumination and the reaction, thus giving rise to a reaction-time. As shown in a previous paper (Folger, 1925), the duration of the reaction-time depends on various factors, such as intensity of the light, previous stimulation, physiological condition of the animal. However, the reaction-time does not constitute the whole of the response to light by amoeba. After stopping, the animal remains motionless for some time, then resumes locomotion. The interval during which movement is suspended and which has been designated the quiescent period or period of quiescence is likewise dependent upon numerous factors and must necessarily be included in a complete investigation of the response to light by the organism.

Investigations dealing with a phase in the reaction of light similar to the one just mentioned have not been numerous. Possibly the growth reaction in a mold, as described by Blaauw (1914), may be compared to it. Blaauw was able by means of mirrors to illuminate the spore-carrier of *Phycomyces nitens* on four sides simultaneously, thus bringing about a symmetrical stimulation and avoiding bending. He found that after an illumination of one minute an increase in the rate of growth could be detected about three and a half minutes later, though this reaction-time was subject to much variation, depending both on the individual and on the intensity of the light. The increase in growth was augmented more and more until in some cases it amounted to as much as two hundred or even four hundred per cent of the normal rate. Shortly afterward, however, growth began gradually to slow down until finally it fell below normal, a condition which was of short duration for soon the plant was growing at its usual rate again.

Apparently more closely related to the phase to be considered in *Amoeba* is that encountered in streaming plant cells such as those of *Chara* and *Nitella*, as described by Ewart (1903), who gives an excellent review of previous work on protoplasmic streaming and adds the results of experiments by himself. Unfortunately for work with illumination, the cells of both of these plants contain chloroplasts, and photosynthesis brings in a complicating factor which interferes somewhat with a study of other effects of light. But in the case of several additional stimulating agents, especially mechanical shock, there is a response similar to that given by *Amoeba* to sudden illumination. The response of a streaming cell of *Chara* to mechanical shock, for instance, comprises a cessation followed shortly by a resumption of movement,

and, as Ewart shows, a close relationship exists between the intensity of the stimulating agent and the time during which streaming remains suspended.

Some use has already been made of the period of quiescence in an investigation of the reactions of *Amoeba*. Folger (1926) demonstrated that in response to mechanical shock it becomes longer with increase in the magnitude of stimulation. This paper is concerned with a study of the quiescent period in the response of *Amoeba* to light.

#### MATERIALS AND METHODS

Apparatus similar to that used in the experiments to be presented has already been described (Folger, 1925). Light was obtained from a 1000-watt, 112-volt, cylindrical Mazda stereoptican lamp and flashed upon the amoebae by means of the plane mirror of the microscope. Heat rays were eliminated by placing a vessel of distilled water between lamp and mirror. The voltage of the current was about 114, and as the amoebae were placed at a distance of 25 centimeters from the light, they were, consequently, subjected to an illumination of approximately 16,000 meter candles intensity, even after some absorption of light by mirror and water. Active specimens of *Amoeba proteus* were used as experimental animals.

#### EXPERIMENTAL RESULTS

Mention has been made of the lengthening of the period of quiescence with increase in the magnitude of mechanical shock. In the experiments referred to, the shock was brought about by dropping pieces of wire through a glass tube to the slide on which the amoebae had been placed. Under these conditions the time of application of the stimulus did not vary, it might be described as instantaneous, and the distance through which the weights fell was not changed. Consequently the intensity of the shocks varied with the weights of the pieces of wire, which were all of the same diameter but of various lengths. In the experiments now to be described, the intensity of the stimulating agent, that is, the light, remained unchanged, but it was easy enough to vary the magnitude of the stimulus by altering the time of exposure. The following effects of light on the period of quiescence were investigated: (1) the effect of changing the length of exposure; (2) the effect of altering the time elapsing between exposures; (3) the effect of repeated exposures.

##### *Relation between the period of quiescence and the exposure period*

If one keeps the intensity of the light constant but alters the time during which it acts, it is immediately apparent that the quiescent period varies with the time of exposure. This is well illustrated by the results of the experiment recorded in Table I, in which an amoeba was subjected to light for periods ranging from 4 seconds to 45 seconds. A response was obtained in every instance, with an average reaction-time of about 2.6 seconds.<sup>1</sup> As can be seen, it was quite otherwise with the period of quiescence. An exposure of 4 seconds produced a quiescent period of 10.6 seconds; one of 10 seconds, a quiescent period of 18.7 seconds; and one of 15

<sup>1</sup> It is not to be assumed that length of exposure can have no effect on the reaction-time, but to produce a reaction-time essentially different from that given above would require a much shorter exposure than any used in this experiment.

seconds, a quiescent period of 24 seconds. Thus the period of quiescence increased with increase of exposure and this continued until the exposure amounted to 45 seconds, when the period of quiescence was 42 seconds.

Further analysis brings out other relationships between exposure and quiescent periods. In the final column is shown the difference obtained by subtracting the former from the latter. With an exposure of 4 seconds the difference amounted to 6.6 seconds. With an exposure of 10 seconds it was 8.7 seconds, and with an exposure of 15 seconds it was 9 seconds. Thus far the rate of increase has been greater in the period of quiescence than in the exposure period, but this did not continue indefinitely, for with an exposure of 20 seconds the difference had fallen to

TABLE I

*Showing increase of the period of quiescence with increase of exposure period. Time indicated by seconds.*

Exposure period (s)	Number of tests	Period of quiescence (t)			t-s
		Maximum	Minimum	Average	
4	5	14	7	10.6	6.6
10	3	20	17	18.8	8.7
15	3	30	21	24.0	9.0
20	3	32	24	27.0	7.0
25	2	29	27	28.0	3.0
30	2	32	32	32.0	2.0
45	1			42.0	-3.0

7 seconds, with an exposure of 30 seconds it was 3 seconds, and with an exposure of 35 seconds it was 2 seconds. The exposure period is now increasing faster than the period of quiescence, and the ultimate result is shown at an exposure of 45 seconds, when the difference amounted to - 3 seconds. In other words, the exposure period has become longer than the period of quiescence. It has already been stated that the amoeba ceased to move after it was in illumination for about 2.6 seconds. So, when exposed for 45 seconds, it stopped in 2.6 seconds and resumed movement in another 42 seconds, 0.4 of a second before the light was turned off. Obviously an exposure greater than 45 seconds would not have called forth a longer period of quiescence, since the amoeba would still have stopped and then resumed movement within about 44.6 seconds after the light was turned on.

#### *Time required for recovery from the effects of sudden illumination*

Reaction by *Amoeba* to light or to mechanical shock is, as previously stated, followed by a refractory period, which may be either absolute or relative; that is, the amoeba may refuse to react to a second stimulus, or it may react but with an altered response. Partial recovery from the effects of either kind of stimulus is indicated by a reaction-time that is longer than that obtained after complete recovery (Folger, 1925, 1926). The effect of partial recovery from sudden illumination on the period of quiescence is illustrated by the results of the experiment shown in Table II.

TABLE II

*Showing the influence upon the period of quiescence on the lack of recovery from the effects of previous stimulation. Light turned on and left on until amoeba resumed locomotion.*

Time in dark previous to exposure	Number of tests	Period of quiescence (sec.)		
		Maximum	Minimum	Average
4 seconds	4	No reaction		
10 seconds	4	30	18	22.5
30 seconds	6	40	15	24.8
1 minute	6	60	25	36.7
2 minutes	6	75	40	54.2
10 minutes	1			175.0
30 minutes	1			220.0
2 hours	1			218.0

In this experiment a single amoeba was used and was subjected to a series of tests in each of which it was first exposed to light for 1 minute, then to darkness for 3 minutes, and again to light, this time for 30 seconds. This procedure was intended to bring about a condition of uniformity. Following the 30-second exposures the light was turned off for intervals varying in the several trials from 4 seconds to 2 hours. Finally in each case the amoeba was then exposed to illumination and left exposed until the characteristic response was completed by the resumption of flow. As shown in the table, no reactions were obtained when 4 seconds were allowed for recovery. Ten seconds permitted a recovery sufficient to obtain reactions in every instance, with an average period of quiescence in 4 trials of 22.5 seconds. Thirty seconds for recovery resulted in an average period of quiescence of 24.5 seconds; 1 minute for recovery, in an average period of quiescence of 36.7 seconds; and 2 minutes for recovery, in an average period of quiescence of 54.2 seconds. Ten minutes, 30 minutes, and 2 hours for recovery were followed by periods of quiescence of 175 seconds, 220 seconds, and 218 seconds, respectively. Clearly the period of quiescence became longer as the amoeba recovered from the effects of previous stimulation, reaching a maximum when recovery was complete. Evidently under the conditions of the experiment complete recovery was not attained until after 10 minutes but before 30 minutes had elapsed. This contrasts with the 1 or 2 minutes necessary to obtain a minimum reaction-time (Folger, 1925, Alsop, 1937).

A few further words of explanation are necessary for a proper evaluation of the results just given. When an amoeba is exposed to light and ceases to move, the stoppage is generally sudden, permitting accurate timing with a stopwatch. If the exposure period is not too long or if too much time has not elapsed between stimuli, the resumption of movement also occurs with sufficient abruptness to be timed with a fair degree of precision. However, when a strong light is combined with sufficient time for complete or almost complete recovery from the effects of previous stimulation and with a long exposure period, especially with one so long that the amoeba begins to move while the light is still acting, the resumption of flow cannot be ascertained with the same accuracy. Under these conditions the first indication of movement is usually an extrusion of a number of small pseudopodia, followed by an erratic flow, first in one direction, then in another. Gradually these

pseudopodia lessen in number, movement becomes stronger, until finally the amoeba is again flowing briskly. If, now, one considers the first indication of movement as the end of the period of quiescence, it is easily determined. But if he assumes that this period is not completed until the amoeba has resumed the more vigorous movement that obtained before stimulation, he must exert a judgement, and the period can not be timed with the same degree of accuracy. In the present instance the period of quiescence was interpreted as ending when the protoplasmic flow became as strong as it was before stimulation, but even if the first indication of movement had been chosen as the final point in the reaction, while the higher figures in the preceding table would have been altered by several seconds, in the very long exposures even by as much as 25 or 30 seconds, the general aspect of a curve drawn from them would not be materially changed.

#### *The effect of repeated stimulation*

Repeated stimulation by light, with a short time intervening between exposures, brings out another peculiarity in the behavior of amoeba, which is exemplified by the results of the experiment summarized in Table III. This table records averages

TABLE III

*Showing decrease in period of quiescence with repeated exposures. Each amoeba subjected to darkness for at least 10 minutes and then given 10 successive 3-second exposures to light, with intervals of 1 minute in darkness between exposures. Number of animals in cultures 1, 2, 3, were 11, 13, and 16, respectively.*

Reaction number	Average period of quiescence (sec.)			General average
	Culture No. 1	Culture No. 2	Culture No. 3	
1	18.5	21.6	14.3	18.1
2	17.8	18.6	14.4	16.9
3	12.6	14.0	14.6	13.7
4	11.6	14.3	12.6	12.8
5	10.4	12.4	12.4	11.7
6	8.8	11.9	10.1	10.3
7	10.8	11.5	11.9	11.4
8	10.4	9.4	10.8	10.2
9	8.8	8.2	9.9	9.0
10	10.3	10.1	8.8	9.7

of the reactions of from 11 to 16 amoebae from each of 3 cultures. Each amoeba was subjected to darkness for at least 10 minutes and then given 10 successive 3-second exposures to light, with intervals of 1 minute in darkness between exposures. Though there were variations among the animals from the same culture and even more among animals from diverse cultures, the general behavior was the same in every case and is reflected in the total averages given in the table. The average period of quiescence for the first exposure amounted to 18.1 seconds, for the second exposure to 16.9 seconds, for the third exposure to 13.7 seconds. As one may see, this decline continued until the sixth exposure, when the period of quiescence was 10.3 seconds. In the 4 succeeding trials at no time did it fall far below 10 seconds

and on the tenth exposure amounted to 9.7 seconds. Even when further tests were added, as they were in some instances though the results are not given in the table, there was no further reduction in the period of quiescence.

This diminution of the period of quiescence with repeated exposure is somewhat reminiscent of the phenomenon known as *treppe*, to be met with in the physiology of muscle.

### DISCUSSION

The response to light that has been under consideration is intimately related to locomotion and must perhaps in final analysis be explained in terms of amoeboid movement. According to Mast (1926), an amoeba consists essentially of an inner fluid plasmasol and an outer solid plasmagel, which is surrounded by a very thin elastic surface membrane, the plasmalemma. The plasmasol is hypertonic and the plasmagel and plasmalemma act as semipermeable membranes, resulting in the development of an osmotic pressure in the plasmasol and a stretching of the plasmagel, especially at these points where it is weakest, where the pseudopodia are formed. Locomotion, according to Mast, involves a continuous change from plasmasol to plasmagel at the anterior end of the animal and from plasmagel to plasmasol at the posterior end. For further analysis of amoeboid movement one is referred to Mast's paper. For our purpose it will suffice to point out that when it is moving, the amoeba must be in a state of dynamic equilibrium. Illumination might cause an increase in the elastic strength of the plasmagel, especially at the tip of the advancing pseudopodium (Mast, 1932), bringing about a temporary breakdown in the established equilibrium, with consequent cessation of forward movement. Shortly an equilibrium is reformed and the animal again moves. Since experiments described in the preceding section have shown that stimulation applied shortly after the resumption of locomotion either fails to bring about a reaction or is followed by a reaction with a shortened period of quiescence, it is apparent that the new equilibrium is by no means at the same level as that which was in existence before the cessation occurred. Otherwise one would expect identical responses when the one stimulation is followed by another of equal strength. The experiments show, moreover, that the original level is gradually restored since a stimulus of a given intensity will finally elicit a second response of exactly the same magnitude as that which it called forth at first. Obviously, the time during which the disturbed equilibrium is reverting to the original level constitutes the refractory period.

### SUMMARY

1. *Amoeba proteus* reacts to sudden illumination by cessation of movement. The time during which it is motionless has been designated the period of quiescence.

2. The period of quiescence becomes longer with extension of the time of exposure to illumination. At first the period of quiescence increases more rapidly than the exposure period, but soon the rate of increase of the latter becomes the greater, with the final result that the amoeba begins to move while the light is still on.

3. Stimulation by light is followed by a refractory period, which may be absolute

or relative; that is, the amoeba may either refuse to respond to a second stimulus, or it may react but with an altered response. Incomplete recovery from the effects of previous stimulation results in a period of quiescence that is shorter than that obtained after complete recovery.

4. If an amoeba is repeatedly exposed to illumination at intervals of a minute, the period of quiescence is at first relatively long, but becomes progressively shorter, arriving at a minimum after about 6 minutes.

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# A METHOD FOR REMOVAL OF THE SINUS GLAND FROM THE EYESTALKS OF CRUSTACEANS<sup>1</sup>

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An increasingly large number of endocrine effects has been attributed to the crustacean sinus gland within the last decade (see reviews by Kleinholz, 1942 and by Brown, 1944). Efforts to determine the role played by these reported endocrine effects in the normal physiology of the animal have proceeded along conventional paths. Although investigators have applied the classical methods of endocrinology in determining in crustaceans the endocrine function of a suspected organ, i.e., deficiency and replacement experiments, the deficiency experiments have not been completely adequate; at times the uneasy feeling arises that some of the reported experimental results may be artifacts.

This situation has come about because removal of the sinus gland, located between the 2nd and 3rd optic ganglia of the eyestalk, is most simply accomplished by ablation of both eyestalks. As has already been pointed out (Kleinholz 1938, 1942), such surgery removes at the same time a not inconsiderable portion of the central nervous system, the four large ganglia located in each stalk. Control experiments to eyestalk ablation, in which antennae or other appendages are removed to simulate equivalent nerve injury or traumatic damage to the experimental animal, are thus not qualitatively comparable. The extent to which these ganglia in the eyestalk are centers of reflex activity, other than visual, has apparently not been adequately determined; the possibility exists that these ganglia of the eyestalk may function as inhibitory centers of nervous activity as does the brain itself (Bethe, 1898; Roeder, 1937).

In recognition of the fact that the most facile surgical procedure is not necessarily the most critical or advisable one, is the growing realization that deficiency experiments in the physiological study of the sinus gland must be made after removal of the sinus gland alone, with as little attendant injury to the nerve ganglia as is possible. Technically, this is not always feasible, especially among the smaller crustaceans. With the larger macrurans and brachyurans, however, surgical excision of the sinus gland is possible by the application of a little ingenuity. Brown (1942) has described such a method for removing the sinus gland from the eyestalk of the crayfish. The procedure described in this note allows removal of the sinus gland with perhaps less disturbance or injury to the eyestalk than the method of Brown. As described here, the method represents the adoption, over several years, of slight modifications in technique to solve the various difficulties that ap-

<sup>1</sup> This work was started in 1941 with the aid of a grant from the Permanent Science Fund of the American Academy of Arts and Sciences. Continuation after the war was made possible by a John Simon Guggenheim Memorial Fellowship. I am indebted to the Chairman of the Division and to the Director of the Biological Laboratories of Harvard University for the many courtesies and facilities extended me.

peared during removal of the sinus gland; other investigators who may attempt such surgery will probably discover alternative improvements in the method.

Such gland removal has been performed on the crayfish and the lobster, the latter animal being easier to operate upon because of its larger size. The steps outlined below apply more readily to the crayfish.

It was early found necessary to have the animal anesthetized, because with an animal that struggled during the more delicately manipulative steps of gland removal, serious damage of the nervous components of the eyestalk would result. Trial of several methods of anesthesia (chloretone, cold) were unsatisfactory; diluted chloroform water proved to be excellent for the purpose. This was prepared by saturating 100 ml. of water with 1 ml. of chloroform by vigorous shaking, allowing the mixture to stand for 2 or 3 minutes so that undissolved chloroform droplets would settle, and pouring the supernatant into a large beaker to which was added about 500 ml. of water. The crayfish was immersed in this dilute chloroform solution for 1-3 minutes; much longer immersion gave too deep anesthesia which was not always followed by successful recovery. After removal from the chloroform solution the animal was washed briefly in running tap water and fastened to an operating board.

The operating board was a rectangular piece of plywood, about  $3\frac{1}{2}$  inches by 6 inches in its dimensions, with a short stout elastic rubber band encircling each end. The anesthetized animal is held in place by inserting the chelipeds under the elastic band at one end, and the abdomen under the rubber band at the other end. After thus fastening the animal, a ligature of cotton thread is loosely tied around the non-skeletogenous base of the eyestalk. This serves as an effective hemostat during later steps of gland removal; excessive bleeding can be prevented or reduced by tightening the ligature. A small piece of modelling clay is used as a wedge between the stalk and the rostral orbit (Fig. 1); this keeps the eyestalk in a fixed position and prevents damage to the retina that might ordinarily result from pressure of the stalk against the carapace or rostral base during subsequent trephining.

Trephination of the exoskeleton is accomplished with the aid of drills of appropriate size. For the eyestalk of *Astacus trowbridgei*, used in this study, a trephine measuring about 2 mm. in outside diameter was satisfactory.<sup>2</sup> The center-pin of the trephine was placed, as shown in Figure 2, slightly posteriorad of the long axis of the stalk and approximately in the center of the non-retinal portion. Rotation of the drill would cut a circumscribed disc of the exoskeleton (Fig. 3). It was found helpful to perform the trephination with the aid of a binocular dissecting microscope, using a magnification of about 18-20 $\times$ , and employing a source of bright illumination such as an adjustable-focus microscope lamp. Once the disc of exoskeletal material had been cut thru, it was removed after carefully separating it from the underlying hypodermis by means of a fine knife. A series of knives of various sizes and shapes was prepared by sharpening steel needles on a hone and mounting them in convenient handles. The excise skeletal disc was placed in normal saline, to be used later in closing the opening. The exposed hypodermis, readily recognized by its numerous red chromatophores, was dissected free and also stored in saline. When this has been done and the bright light focused on the operative field, the nervous portion of the eyestalk and the outline of the sinus gland can

<sup>2</sup> Drills of assorted sizes have been made for me by Mr. H. G. Bliss, 507 Park Avenue, Herkimer, New York.

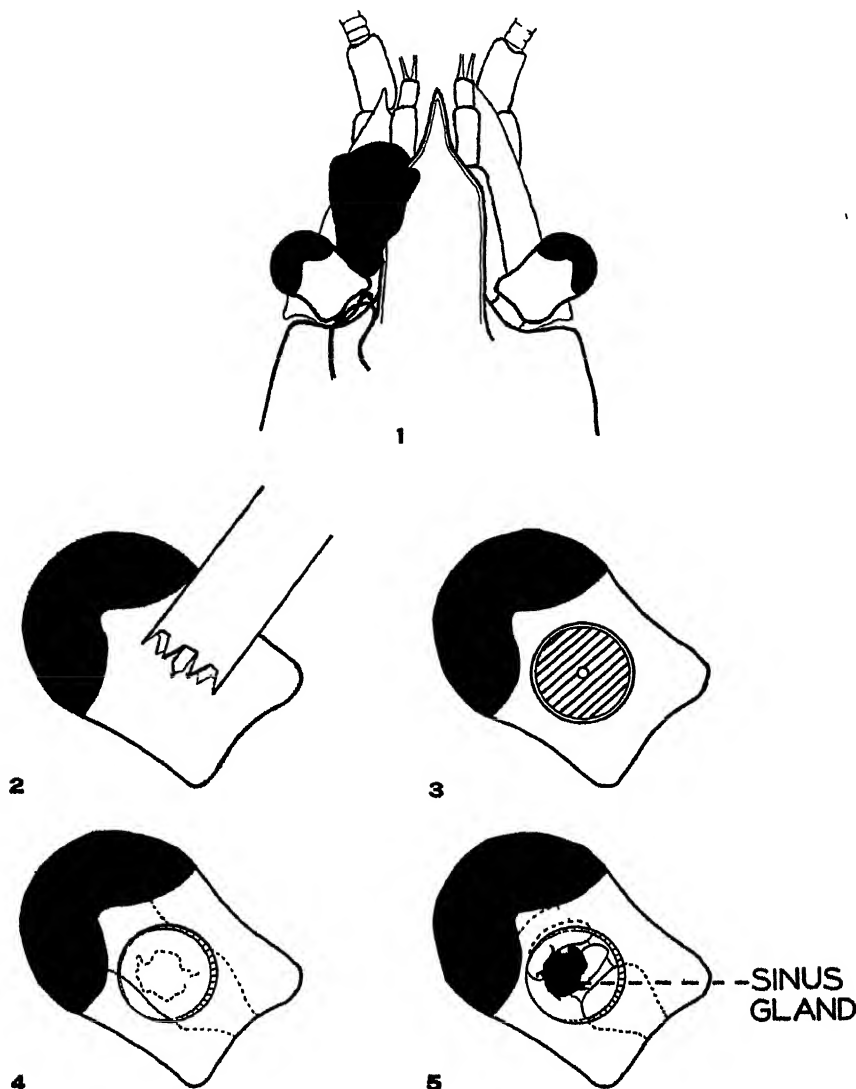


FIGURE 1. The cephalic end of a crayfish, showing the left eyestalk prepared for removal of the sinus gland. A ligature is first loosely tied around the base of the eyestalk; this is tightened only before trephination is started. A piece of modelling clay holds the stalk in position and avoids the risk of crushing the retinal portion against the rostrum or carapace.

FIGURE 2. The left eyestalk viewed from the dorsal side, showing the location of the trephine to expose the area of the sinus gland.

FIGURE 3. The trephined area of the exoskeleton shown in diagrammatic form.

FIGURE 4. The trephined disc of skeletal material has been removed; the hypodermis, lying directly underneath the exoskeleton has also been excised. In dotted outline are shown the optic ganglia and the sinus gland, enclosed in a thin transparent sheath. The muscles of the eyestalk are not shown.

FIGURE 5. The connective tissue sheath has been removed, showing the gland and its processes located between the 2nd and 3rd optic ganglia.

be detected with the binocular microscope. Some of the muscles of the stalk lie alongside the nerve ganglia, but can be readily separated from the latter by a fine knife. The operative field at this point is diagrammed in Figure 4. A thin, transparent sheath encloses the optic ganglia and the sinus gland. This sheath is carefully slit, and the incision extended around the margin of the trephined area. In the light of the microscope lamp the sinus gland now appears as a bluish-white structure, quite easily distinguishable from the nervous portion of the stalk (Fig. 5).

The sinus gland is located between the second and third optic ganglia, in a somewhat dorso-posterior position with regard to the optic tract. In the crayfish (*Cambarus* and *Astacus*) it is not as compact a structure as is found in many of the marine crustaceans; a few filaments or processes of the gland are found to extend to adjacent ganglia; in the eyestalk of *Astacus* this seems to be less pronounced than in *Cambarus*.

After the gland has been exposed, the processes and body of the gland are carefully separated from the optic ganglia by a knife, and the entire structure removed with the aid of watchmaker's forceps and iridectomy scissors. The operative field is examined with the binocular microscope to verify complete excision; fragments of the filaments that have remained behind can be removed with the forceps.

Following removal of the sinus gland, the hypodermis, previously excised and saved in saline, is replaced to fill the gap and the trephined disc of skeletal material is fitted into the opening in the exoskeleton of the eyestalk. The groove between the margin of this opening and the rim of the disc is filled with fibrin foam. This material originally consisted of samples from the laboratory of Professor E. J. Cohn of the Harvard Medical School; later, commercial preparations, "Gelfoam, No. 12" manufactured by the Upjohn Company, proved equally satisfactory. The ligature around the base of the eyestalk is removed. The slight oozing of blood that follows in the trephined area soon leads to clotting in contact with the fibrin foam. To facilitate this clotting and the desired fixing-in-place of the skeletal disc, the animal is removed from the operating board and placed in a vessel containing water to a depth not much greater than an inch; after 2-3 hours the operated animal is replaced in its tank of deeper water, with little risk that the trephined disc will be loosened and the area of operation exposed.

In practice I have found that the operation from anesthesia to gland removal and replacement of the disc requires about 15 minutes. The initial attempts required as long as an hour, until some facility in the procedure was attained.

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# THE COMPARATIVE TOLERANCES OF SOME FOULING ORGANISMS TO COPPER AND MERCURY \*

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The ability of marine organisms to attach to surfaces coated with ship-bottom paints depends on the toxicity of the surface and the sensitivity of the organisms to the toxic. Pyefinch (1946) presents a table showing the sensitivity of various organisms to toxics determined primarily from analysis of their settlement on toxic surfaces at Millport, Scotland. In the present studies carried out in the Biscayne Bay area, Florida, the tolerances of several common fouling organisms to copper and mercury have been compared by observations of the sequence of attachment to antifouling paints of moderate toxicity, to series of paints of graded toxicity, and to nontoxic surfaces adjacent to antifouling paint.

## RESULTS

An indication of the relative tolerance of various organisms to toxic paints can be obtained by observing the frequency of their attachments to paints which are beginning to fail after long exposure.<sup>1</sup> It may be expected that the most tolerant forms will be the first to attach. The organisms attaching earliest to each of 662 antifouling paints exposed at Miami Beach and Tahiti Beach, Florida, were recorded with the following result:

Barnacles	67%
Encrusting bryozoans	18
Hydroids	9
Erect bryozoans	5
Tunicates	1
Tubeworms	< 0.1
Sponges	< 0.1

The order of attachment depends not only upon the tolerance of the organisms to the toxics, but also upon their seasonal occurrences. For example, hydroids and those forms following them in the list attach first only when barnacles and encrusting bryozoans are absent from the fouling population. Encrusting bryozoans rank below barnacles because they are absent during a large part of the year, while barnacles are present at almost all times. One of the encrusting bryozoans, *Water-sipora cucullata*, appears to be somewhat more tolerant to copper than the most tol-

\* Contribution No. 385 from the Woods Hole Oceanographic Institution. The observations described were made during an investigation of fouling by the Woods Hole Oceanographic Institution under contract with the Bureau of Ships, Navy Department, which has given permission for their publication. The opinions presented here are those of the author and do not necessarily reflect the official opinion of the Navy Department or naval service at large. The author is indebted to Dr. B. H. Ketchum and Dr. A. C. Redfield for helpful advice and criticism.

erant barnacle species, *Balanus amphitrite*, since it frequently attached earlier when both organisms were present.

The larva of a fouling organism which is unable to attach to a toxic paint surface may attach to a more resistant form which has already become established on the surface. Following this the adult may then spread over the paint surface. The relative tolerances of some fouling organisms to the toxic surfaces can frequently be assessed from these conditions of attachment.

The barnacles, *Balanus amphitrite* and *Balanus improvisus*, and the encrusting bryozoan, *Watersipora cucullata*, were found attached directly to paint surfaces. Attaching to and growing on these forms were *Schizoporella unicornis*, *Anomia* sp., *Bugula avicularia*, *Pennaria tiarella*, *Lepas anatifera* and tunicates. Examples of several of these organisms which were unable to attach to antifouling paints, but which attached to other forms on the paint, are shown in Figures 1-5.

A more precise evaluation of tolerance is given by comparing the frequency of attachment of different organisms on a surface of moderate toxicity with their frequency on a nontoxic control surface. A paint of moderate toxicity will allow attachment only of the forms having the highest tolerance to the paint toxics. Glass panels coated on one side with a paint in which cuprous oxide served as the toxic were exposed for periods of one month. The uncoated side served as a control. The use of the glass panel gave both surfaces the same color, thus eliminating any differential effect of color on attachment. Table I shows the numbers of barnacles

TABLE I

Total number of barnacles attached to 33 nontoxic and toxic surfaces (80 square inches in area) exposed for one month at intervals between October 1944 and May 1946

Barnacle species	Barnacles on nontoxic surface		Barnacles on toxic surface	
	Total	% of total	Total	% of total
<i>Balanus improvisus</i>	17,959	89.7	15	9.4
<i>Balanus amphitrite</i>	1,503	7.5	145	90.6
<i>Balanus cburneus</i>	564	2.8	0	0.0

of three species collected on these two surfaces. The total numbers of barnacles attaching to the toxic paint surface are less than one per cent of those growing on the nontoxic control. *Balanus amphitrite* comprises over 90 per cent of the population on the toxic surface, being the only barnacle species on most panels, though it is only 7.5 per cent of the population attaching to the control surface. In contrast *Balanus improvisus* makes up 90 per cent of the total attachment to the nontoxic surface but comprises only 9.4 per cent of the population on the paint. *Balanus cburneus* is completely inhibited from the toxic surface. It is thus evident that each of these three species of the same genus demonstrates a different degree of tolerance to the copper paint.

In addition to the barnacle fouling the only other significant attachment to this paint surface was the encrusting bryozoan *Watersipora cucullata* and the green alga *Enteromorpha* sp. Both of these species attached only during the periods of their maximum abundance. On the nontoxic panels a total of twenty species of fouling

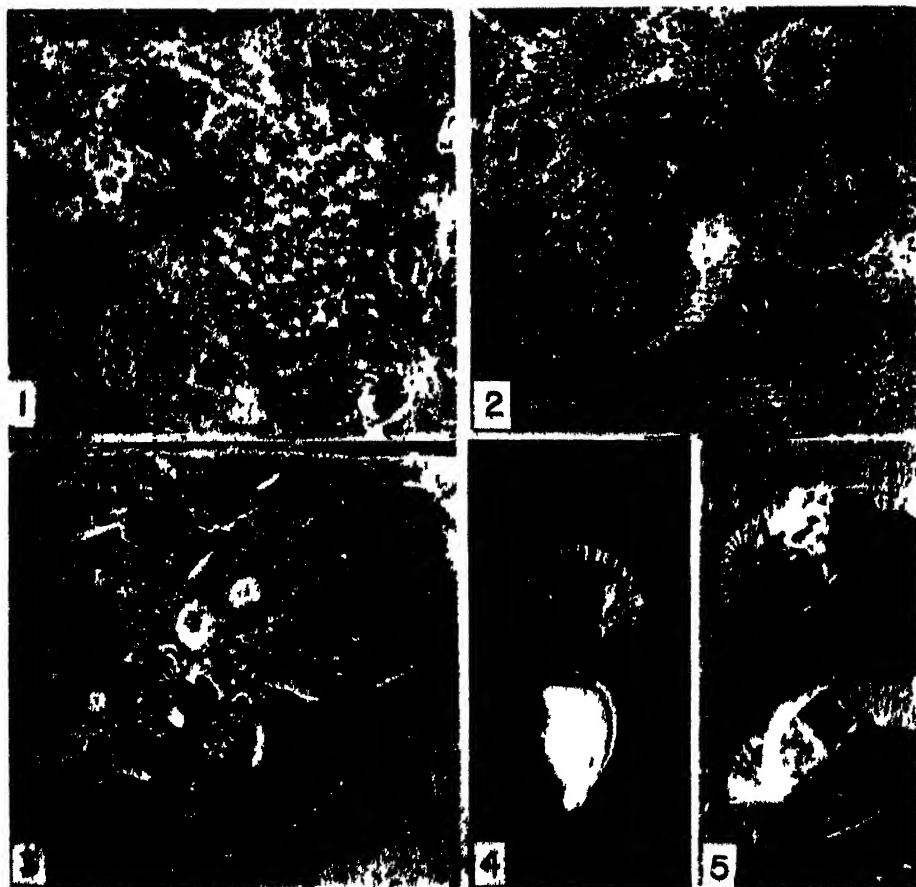


PLATE I

FIGURE 1. A speckled tunicate limited in attachment to barnacles and *Inomia* sp. without spreading onto the paint surface.

FIGURE 2. *Bugula avicularia* attached to the encrusting bryozoan, *Watersipora cucullata*, which is growing directly on the paint.

FIGURE 3. Barnacles attached to *Watersipora cucullata* which developed directly on the paint surface. Tubes of *Dasychone conspersa* are also attached on *W. cucullata*.

FIGURE 4. Gooseneck barnacle, *Lepas anatifera*, attached to *Balanus amphitrite* which attached to an otherwise effective antifouling paint on a ship's bottom.

FIGURE 5. Two examples of *Balanus improvisus* which attached to the more resistant barnacle, *B. amphitrite*. Photographed after removal from the paint surface.

organisms attached during the period covered by these observations. These must be considered to be less tolerant than the four organisms which attached to the paint surface.

In order to establish the relative tolerances to copper of the more sensitive fouling forms a set of paints containing graded amounts of copper flake pigment was prepared. The copper leaching rates of these paints were measured as described by

Ketchum, Ferry, Redfield, and Burns (1945) and were found to vary in proportion to the pigment content and to decrease with the time of exposure. This series thus provided graded toxicities.

The relative tolerance of attaching organisms could be judged by the time of exposure elapsing before they attached to any one member of the series or by the number of members of the series which were fouled at any one time. The paints were applied to 8 × 10-inch weldwood panels which were immersed at Miami Beach and Tahiti Beach, Florida. The composition of the paints and the time of exposure before various fouling forms attached are given in Table II.

TABLE II

Number of months of exposure prior to appearance of fouling on painted panels immersed in the sea at Miami Beach, Florida (May 2, 1945–March 2, 1946), and at Tahiti Beach, Florida (May 2, 1945–September 2, 1945). The paints contained graded amounts of copper flake in a vehicle consisting of equal parts, by weight, of polyvinyl butyral and rosin.

Paint number Copper content, per cent dry weight	AF12 90	121 67	122 45	123 30	124 23	125 14
Miami Beach						
<i>Balanus amphitrite</i>	4	3	3	2	1	1
<i>Watersipora cucullata</i>	9	9	8	2	2	1
<i>Balanus improvisus</i>	a	a	a	a	2	1
Tahiti Beach						
<i>Polysiphonia</i> sp.	3	2	2	2	2	2
<i>Balanus amphitrite</i>	b	b	4	4	3	1
<i>Hydroides parvus</i>	b	b	b	4	4	3

a. None of this species had attached to these paints after 10 months of exposure.

b. None of these species had attached to these paints after 4 months of exposure.

At Miami Beach only three fouling organisms attached to the copper paints, two species of barnacles and the encrusting bryozoan *Watersipora cucullata*. Throughout the period of exposure there was no marked seasonal variation in the incidence of barnacles. However, seasonal fluctuation in the incidence of *Watersipora cucullata* accounts principally for the nonattachment of this organism between the second and ninth months of immersion of the paints. Its absence from the paints is, therefore, not indicative of its sensitivity to the paint toxic.

The exposure at Tahiti Beach, Florida, indicated that, on these copper paints, a red alga of the genus *Polysiphonia* is more tolerant, and that the tube worm, *Hydroides parvus*, is less tolerant than *Balanus amphitrite*.

Some species of fouling were too sensitive to attach even to the paints of lowest toxicity. To ascertain their relative tolerance of paint toxics their sequence of attachment was noted on nontoxic areas adjacent to paint surfaces. Observations by Miller (1946) on the diffusion of toxics from antifouling paints indicate that toxic gradients exist, decreasing from the paint edge. To provide nontoxic areas for attachment adjacent to the paint surfaces, strips 5, 10, and 20 mm. in width were left free of paint on the panels of the graded toxicity series.

At both exposure locations colonial tunicates attached to the widest bare strips on the paints of lowest toxicity, AF124 and AF125, by the second month of immersion.



By the third and fourth months of exposure hydroids had attached to the initial fouling on these nontoxic areas. These observations indicate that tunicates are more tolerant than hydroids to copper but less tolerant than those forms able to attach directly to the paint surface.

From the preceding observations several fouling forms can be arranged in order of decreasing tolerance to copper as follows: *Polysiphonia* sp., *Watersipora cucullata*, *Balanus amphitrite*, *Balanus improvisus*, *Balanus crenatus*, *Hydroides parvus*, tunicates and hydroids.

In the examination of antifouling paints employing mercury pigments or mixtures of mercury and copper pigments as the toxic ingredients, it was noted that certain of the fouling organisms appeared to be more sensitive to mercury than to copper while for others the reverse was true.

To establish the comparative tolerance of different species to mercury a series of paints containing mercurous chloride in graded concentration was made. The vehicle was the same as that used for the copper series. The mercury content of these paints was equal in weight to the corresponding member of the copper series. These paints were immersed simultaneously and at the same locations as the copper paints. The extent of exposure before fouling attached to the paint surfaces of the AF12 mercury series is shown in Table III.

TABLE III

Number of months of exposure prior to appearance of fouling on painted panels immersed in the sea at Miami Beach, Florida (May 2, 1945–March 2, 1946), and at Tahiti Beach, Florida (May 2, 1945–September 2, 1945). The paints contained graded amounts of mercury, added as mercurous chloride, in the vehicle described in Table II.

Paint number Mercury content, per cent dry weight	AF12 90	121 67	122 45	123 30	124 23	125 14
Miami Beach						
<i>Balanus amphitrite</i>	9	9	2	2	2	1
<i>Balanus improvisus</i>	a	a	a	4	9	2
<i>Bugula neritina</i>	a	a	a	2	6	1
<i>Watersipora cucullata</i>	a	a	a	6 <sup>†</sup>	a	4 <sup>†</sup>
Tahiti Beach						
<i>Polysiphonia</i> sp.	b	b	b	3	3	1
<i>Balanus amphitrite</i>	b	b	b	b	2	2

a. None of these species had attached to these paints after 10 months of exposure.

b. None of these species had attached to these paints after 4 months of exposure.

<sup>†</sup> Single specimen not present on following month.

At Miami Beach four organisms attached to this series of paints. The most resistant of these was *Balanus amphitrite*, followed in order by *B. improvisus* and *Bugula neritina*. *Watersipora cucullata*, which was found to be quite tolerant of copper, attached to only two of these paints, and in neither case was the attachment secure. In contrast, *Bugula neritina* was able to attach directly to the mercury paints but was never found on any of the comparable copper paint surfaces. At Tahiti Beach *Polysiphonia* sp. was found to be more tolerant than *Balanus amphitrite* to the mercury paints.

Photographs of two of the copper and two of the mercury paints of these series, after nine months of exposure at Miami Beach, are shown in Figure 6. The ability of *Watersipora cucullata* to attach to the copper paints and its absence from the comparable mercury paints are shown. Both of the copper paints and one of the mercury paints are fouled with *Balanus amphitrite*.

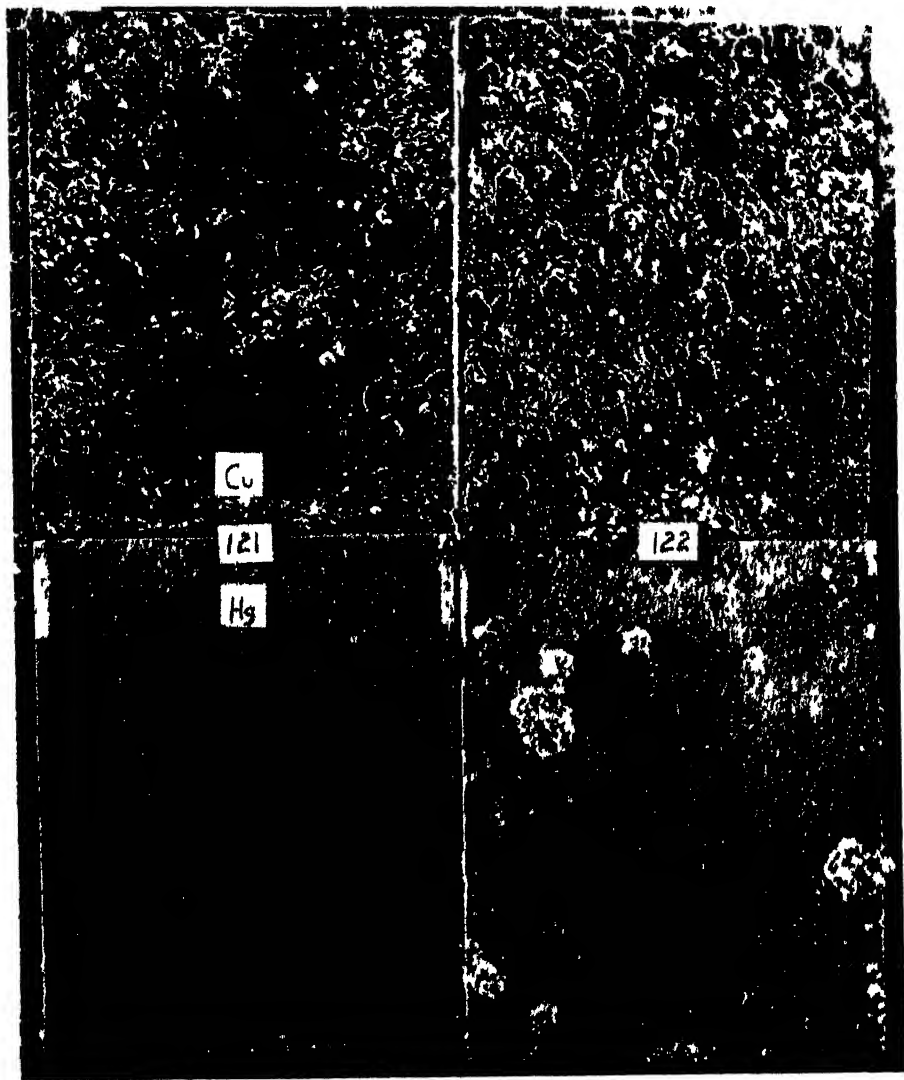


PLATE II

FIGURE 6. Comparison of the attachment of *Watersipora cucullata* to paints pigmented with copper and mercury after nine months of exposure. *W. cucullata* appears on the copper series (top panels) as the dark disc-like organisms.

Nontoxic strips on panels otherwise coated with the mercury paints permitted attachment of the more sensitive fouling forms. The more resistant forms mentioned above always attached first to these nontoxic areas. In addition, *Watersipora cucullata*, *Anomia* sp., hydroids and tunicates invaded the unpainted areas at Miami Beach. At Tahiti Beach *Enteromorpha* sp. and *Hydroides parvus* attached in the order given.

These exposures indicate the following order of decreasing tolerance of some fouling organisms to mercury as a paint toxic: *Polysiphonia* sp., *Balanus amphitrite*, *Bugula neritina*, *Balanus improvisus*, *Watersipora cucullata*, *Anomia* sp., *Enteromorpha* sp., *Hydroides parvus*, hydroids and tunicates.

From the results of the various methods described above the comparative tolerance of several fouling organisms to copper and mercury has been evaluated. The results are given in Table IV. The organisms listed as attaching to adjacent

TABLE IV

The comparative tolerance of some fouling organisms of the Biscayne Bay area, Florida, to copper and mercury employed as pigments in antifouling paints. The lists are arranged in approximate order of decreasing tolerance.

	Copper	Mercury
Organisms found attached to toxic paint surfaces	<i>Polysiphonia</i> sp. <i>Watersipora cucullata</i> <i>Balanus amphitrite</i> <i>Enteromorpha</i> sp. <i>Balanus improvisus</i> <i>Balanus eburneus</i> <i>Hydroides parvus</i>	<i>Polysiphonia</i> sp. <i>Balanus amphitrite</i> <i>Bugula neritina</i> <i>Balanus improvisus</i> <i>Watersipora cucullata</i>
Organisms found mainly attached to nontoxic areas adjacent to paint surfaces	<i>Anomia</i> sp. <i>Bugula neritina</i> <i>Bugula avicularia</i> <i>Lepas anatifera</i> tunicates hydroids	<i>Anomia</i> sp. <i>Enteromorpha</i> sp. <i>Hydroides parvus</i> hydroids tunicates

nontoxic surfaces include those which may on occasion be found on paints of low toxicity but which generally do not attach directly to the paint surface. The exact position in the order of decreasing tolerance, for all but the first three organisms in each column, requires further verification.

### DISCUSSION

Relatively few of the sessile species in the Miami area which attach to neutral surfaces are able to attach to antifouling paints. Even paints of such low toxicity that they become completely covered permit the attachment of only four or five forms. Many less resistant fouling organisms are excluded from the paint surface. Effective antifouling paints must be designed to prevent the attachment of the few most tolerant forms.

The species most tolerant of copper are different from those most resistant to mercury. The conflicting opinions which have existed concerning the relative

efficiencies of copper and mercury compounds as paint toxics may depend largely on the species of fouling organisms present at the particular testing location.

The present results and those given by Pyefinch (1946) agree in showing that *Enteromorpha* and *Polysiphonia* are among the more resistant forms, and *Anomia* and tunicates have low tolerance to paint toxics. Tubularian hydroids were noted only as secondary fouling at Miami, though Pyefinch rates them more resistant than *Balanus* and equal to *Enteromorpha*. A direct comparison of the results with *Balanus* is not feasible since the three species found in Miami showed marked differences in toxic sensitivity. None of these species are found at Millport where Pyefinch's studies were made. Pyefinch lists a greater variety of algae, including diatoms, which were not included in the investigations at Miami.

#### SUMMARY

The comparative toxic tolerance of several fouling organisms was based on the sequence of their attachment to copper and mercury antifouling paints. A red alga, *Polysiphonia* sp., and the barnacle, *Balanus amphitrite*, were the most tolerant and attached to copper and mercury paints before other organisms. The encrusting bryozoan, *Watersipora cucullata*, was found to be slightly more tolerant to copper but considerably less tolerant to mercury than the above two forms. Less tolerant forms were *Balanus improvisus*, *Hydroides parvus*, *Bugula neritina*, *Anomia* sp., *Enteromorpha* sp., tunicates and hydroids. Other species, though attaching to nontoxic surfaces, were never found on the toxic paints.

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# SYSTEMATIC SEROLOGY AMONG CERTAIN INSECT SPECIES

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## INTRODUCTION

The measure of the biochemical similarity among organisms is of interest to all persons doing research whose ultimate goal is to determine the essential nature of organisms. A natural consequence of studies in this direction is the systematic classification of organisms based on the similarities and differences in their serological behavior. The blood sera and the soluble protein constituents have been the chief substrates through which serological classifications have been made. Extractions of whole organisms such as insects have also proved to be adequate for comparing the similarity among insect species (Leone, 1947). Systematic serology does not necessarily answer questions on the phylogenetic origin of organisms, but rather associates animals as they exist today into natural classifications based on their essential biochemical similarities.

The following paper is designed to show the type of serological results that can be expected when various families in the insect order Orthoptera are examined serologically. The organisms used in these tests are listed below:

Family	Scientific name	Common name
Acrididae	<i>Melanoplus femur-rubrum</i> DeG.	Red-legged grasshopper
	<i>Melanoplus differentialis</i> Thos.	Differential grasshopper
	<i>Romalea microptera</i> Beauv.	Florida lubber grasshopper
	<i>Paroxya atlantica</i> Scudd.	Atlantic locust
	<i>Leptysma marginicollis</i> Serv.	Slender locust
	<i>Spharagemon bolli</i> Scudd.	Boll's locust
	<i>Arphia xanthoptera</i> Burni.	Yellow-winged locust
Tettigoniidae	<i>Conocephalus strictus</i> Scudd.	Straight-lanced grasshopper
	<i>Conocephalus fasciatus</i> DeG.	Slender-meadow grasshopper
Mantidae	<i>Paratenodera sinensis</i> Sauss.	Chinese mantis
Gryllidae	<i>Gryllus assimilis</i> Fabr.	Field cricket
Blattidae	<i>Periplaneta americana</i> (Linn.)	American cockroach

## MATERIALS AND METHODS

### *Preparation of antigens*

After first removing the tarsi and the wings the insects were ground in a mortar with sand. Sufficient buffered 0.85 per cent NaCl was added to prevent drying and denaturation of the saline soluble components. The resulting emulsions were cleared using the same techniques described in a previous paper by the author (Leone, 1947). Micro-Kjeldahl nitrogen determinations were performed to determine the total nitrogen and the non-protein nitrogen. Values for the protein content of the antigens were determined as follows: grams total nitrogen — grams non-protein nitrogen = grams protein nitrogen  $\times 6.25 \times 100$  = grams per cent pro-

tein in solution. These values are given in Table I. When only a few insects of a given species were available, it was necessary to dilute the extract sufficiently to obtain volumes large enough to conduct the projected tests. As a result of this the concentrations of protein in solution were markedly reduced in some antigen extracts.

TABLE I  
*Protein concentrations in extracts of Orthoptera*

Organism	Key	Grams protein per cent
<i>Melanoplus femur-rubrum</i>	GR	0.32
<i>Melanoplus differentialis</i>	GD	0.22
<i>Romalea microptera</i>	GF	0.07
<i>Paroxya atlantica</i>	GA	0.05
<i>Leptysma marginicollis</i>	GS	0.03
<i>Spharagemon bolli</i>	GB	0.10
<i>Arphia xanthoptera</i>	GY	0.06
<i>Conocephalus strictus</i>	GL	0.04
<i>Conocephalus fasciatus</i>	GM	0.05
<i>Paratenodera sinensis</i>	MC	0.09
<i>Gryllus assimilis</i>	KC	0.13
<i>Periplaneta americana</i>	CA	0.82

#### *Preparations of antisera*

A series of four doubling doses of antigen were injected intravenously in rabbits on alternate days; initial injection was one ml. A second series of antigen injections were given to a rabbit if a preliminary bleeding and testing seven days after the last injection revealed little or no response to the antigen. Final bleedings by cardiac puncture were performed on the eighth day after the last injection. The blood was permitted to clot for 24 hours. The expressed sera were centrifuged, sterile filtered through a Seitz filter, bottled in serum vials, and stored in the refrigerator.

#### *Methods of testing*

Ring tests were performed in accordance with standardized procedures as outlined by Boyden (1926).

Photoelectric measurements of precipitin turbidities were made using the Libby photoreflexometer (photron'er) (1938). Titrations were performed and results plotted using the technique of Boyden and DeFalco (1943).

### EXPERIMENTAL RESULTS

The production of immune sera against all the Orthoptera except the Blattidae (roaches) proved to be difficult. Protein concentrations were low, and this fact, together with the small quantities of material available restricted the number of injections that could be used to produce an antiserum, and also restricted the number of tests that could be carried out with any one antigen. The photron'er curves proved to have low peaks and broad bases. Inter-family relationships could be established with these curves. Ring test reactions, in general, confirmed the whole curve comparisons of the insect antigens, in so far as this limited technique can be

compared with the nephelometric photron'er method. Typical sets of data are presented to show the correspondence obtained by the two methods (Figs. 1 and 2). A summary of all the information obtained using both techniques is presented in Table II.

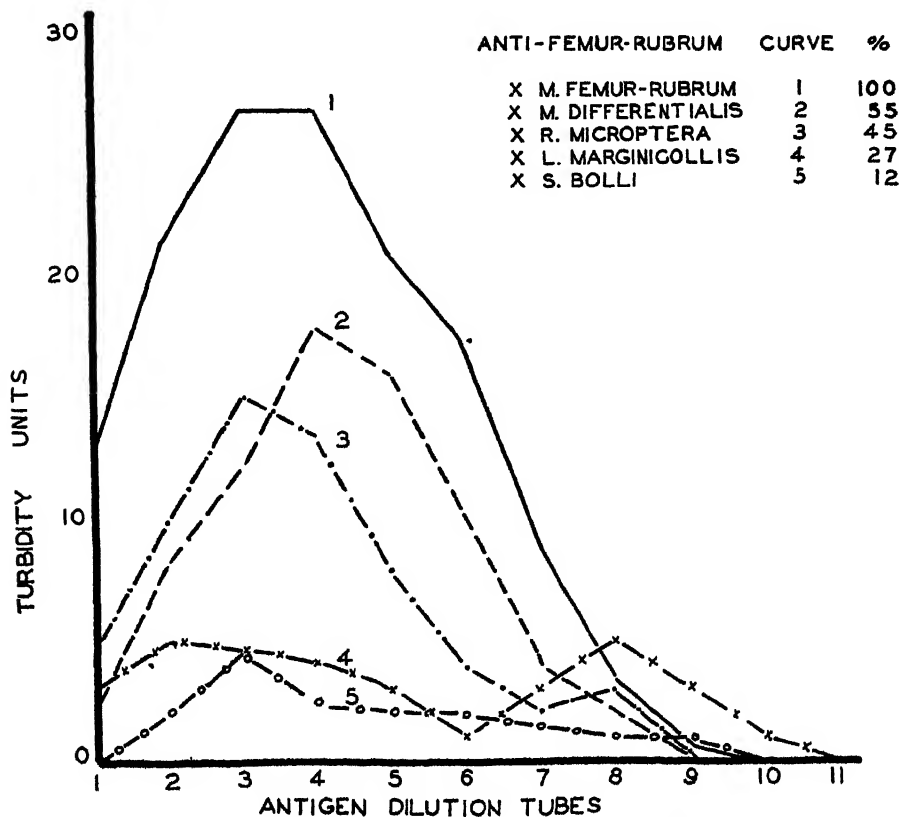


FIGURE 1. A photron'er curve series titrated against an anti-*Melanoplus femur-rubrum* serum. Percentage values indicate the relative degrees of correspondence to the homologous antigen *Melanoplus femur-rubrum*, demonstrated by the various heterologous antigens. The undiluted antiserum shows a low reactivity but a broad reactivity range.

#### *Family Acrididae (Short-horned grasshoppers)*

Antisera sufficiently powerful to be recorded on the photron'er were secured in only two instances, one against the Florida Lubber grasshopper (*Romalea microptera*), and the other against the Red-legged grasshopper (*Melanoplus femur-rubrum*). The latter antiserum was obtained as the result of a double series of injections separated by a period of eight days between the first and second injection series.

The anti-Lubber grasshopper serum showed only a slight discrimination between the Red-legged and the Differential grasshoppers (*Melanoplus differentialis*).

TABLE II  
Comparison of ring test and photron'er test results

Antisera	Homologous titer in thousands	Antigens (Relationship values in per cent)											
		Red-legged grasshopper	Differential grasshopper	Florida lubber grasshopper	Atlantic locust	Boll's locust	Yellow-winged locust	Slender locust	Slender-meadow grasshopper	Straight-lanced grasshopper	Chinese mantis	Field cricket	American roach
Photron'er Tests		100.0	54.9	50.7	44.7		29.2	23.8		12.4	0.0	0.0	0.0
		13.5	9.6	100.0			0.0		0.0	0.0	0.0	0.0	0.0
		0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0	0.0	100.0	6.2	18.7
		6.0	4.5			7.3	0.0		0.0	0.0	10.5	100.0	29.0
		0.0	0.0						0.0	0.0	0.0	4.3	100.0
Ring Tests		100.0	50.0	25.0	50.0		50.0	50.0		12.5	6.8	12.5	6.8
		32	100.0	100.0	50.0		0.0		0.0		0.0	0.0	0.0
		64	12.5	3.4	100.0	0.0	0.0	0.0			0.0	0.0	0.0
		32	100.0		100.0	0.0	0.0	0.0	0.0	25.0	100.0	25.0	25.0
		256	0.0	0.0	0.0	0.0	0.0	0.0		12.5	6.8	100.0	25.0
		256	6.8		0.0	0.0	0.0	0.0		12.5	6.8	25.0	100.0
		256	6.8	6.8	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0
		128	100.0	50.0	50.0		25.0	25.0		0.0	0.0	0.0	0.0



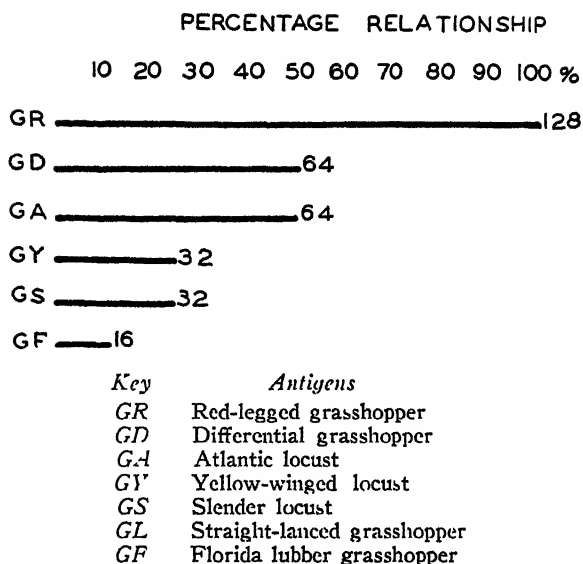


FIGURE 2. A typical plot of ring test titers obtained by heterologous antigens titrated against an anti-*Melanoplus femur-rubrum* serum. The number behind each line represents the highest dilution, in thousands, at which a definite interfacial ring was obtained. Ring tests performed using undiluted antiserum gave undifferentiating titers among all the *Acrididae* except the Lubber grasshopper. A dilution of the antiserum with equal parts of saline gave the above discrimination.

The anti-Red-legged grasshopper serum demonstrated an ability to react against many heterologous antigens. Even closely related forms could be readily distinguished, however of the species that showed reactivity, the Straight-lanced grasshopper (*Conoccephalus strictus*) belonging to the more distant Tettigoniidae, showed the least amount of turbidity. The mantids, crickets, and roaches produced no heterologous turbidities in the photron'er and thus indicated their more distant relationship.

The confirmation of the reduced reactivity of these more distant species when tested with the ring test was striking. Intra-family differences could not be demonstrated with the ring test (exception may be cited in the case of the Lubber grasshopper *Romalea microptera*). A dilution of one part serum to one part saline reduced the reactivity of the antiserum to such an extent (and also increased its specificity) that all organisms outside the family no longer reacted.

Antisera procured against other members of the family Acrididae, i.e., the Differential grasshopper and the Atlantic locust were too weak to give photron'er readings. Ring test titers for these organisms were very low also, the homologous reactions having a titer of only 1 to 32000.

#### *Family Mantidae (Praying mantids)*

Only a single species of this family, the chinese mantis (*Paratenodera sinensis*) was secured. The photron'er results indicate a very specific antiserum with the

families Blattidae and Gryllidae barely making their appearance. The Blattidae (*Periplaneta americana*) show a closer relationship to the Mantidae than any other family considered.

Ring test results indicate undifferentiating heterologous titers among the cricket, roach, and Straight-lanced grasshopper, representing their respective families Gryllidae, Blattidae, and Tettigoniidae when compared against the Mantid. The family Acrididae, evidently more distant, did not react.

#### *Family Gryllidae (Crickets)*

In view of the morphological indistinctness of the several large black species of crickets, all the specimens collected were pooled and treated as a single type species (*Gryllus assimilis*) to represent this family. By photron'er test the Blattidae (roaches) showed the closest relationship; other families, on the whole, reacted weakly. Greater differentiation of these distant relatives was shown by the ring tests.

#### *Family Tettigoniidae (Long-horned grasshoppers and katydids)*

Two species were secured to represent this family, the Straight-lanced grasshopper (*Conocephalus strictus*) and the Slender-meadow grasshopper (*Conocephalus fasciatus*). Antisera were not obtained against these antigens. Both species were used however in the heterologous reactions to aid in establishing their position with respect to other species and the position of other species with respect to them.

#### *Family Blattidae (Roaches)*

One sample of the American cockroach (*Periplaneta americana*) was chosen to represent this family. The antiserum was the most powerful of all those tested, i.e., gave the greatest turbidity readings, but also was among the most specific of the antisera with respect to inter-family reactions on the photron'er. The field encompassed by means of the ring test technique is somewhat broader than the photron'er examination of this antiserum. The Gryllidae reactions were the most nearly like the Blattidae, of all the families tested. The Tettigoniidae, Acrididae, and the Mantidae showed lessened but approximately the same degrees of relationship to the Blattidae.

### DISCUSSION

The results as presented above are at best only a beginning in the study of the problem of the quantitative systematic serology of insects. The insect species used in the tests were chosen because of their availability to the writer. No attempt was made to choose species in such a way, or to perform tests in such a manner as to solve particularly significant problems in insect relationships. It was desired to know the type of serological results that could be obtained using extracts of whole insects as antigens characteristic for the species tested.

The results do indicate the feasibility of making a quantitative serologic analysis of representatives of the families, genera, and species of insects.

Boyden (1943) has been able to establish relatively constant inter-species, inter-generic, and inter-family relationships among the Crustacea. His average value for

the relationship of sera of the same genus is 46 per cent; the averaged value for relationships of genera in the same family is 30 per cent; and, for inter-family relationships the average is 9 per cent.

Intra-generic values for the two species of *Melanoplus* considered in this paper have a value of 54.7 per cent. The averaged relationships of extracts of genera within the insect family Acrididae proved to have a value of 28 per cent. Inter-family relationships for the order Orthoptera have an average value of 11.5 per cent. The extremes of the serological relationship values for any given group using extracts of fresh insects are all within the extreme values reported by Boyden, who used whole sera for his comparisons. The averaged values obtained for relationships between species, genera, and families are in accord with the Crustacea values.

More information will have to be acquired on the nature of serological reactions, and more data accumulated on actual systematic tests before serological limits in terms of percentages, or other statistical rankings, can be summarized or defined as representing "species," or "genera," or "families" of insects.

Tentative serological relationships for four of the five families of Orthoptera investigated are given in Figure 3. The fifth family tested, the Tettigoniidae are not

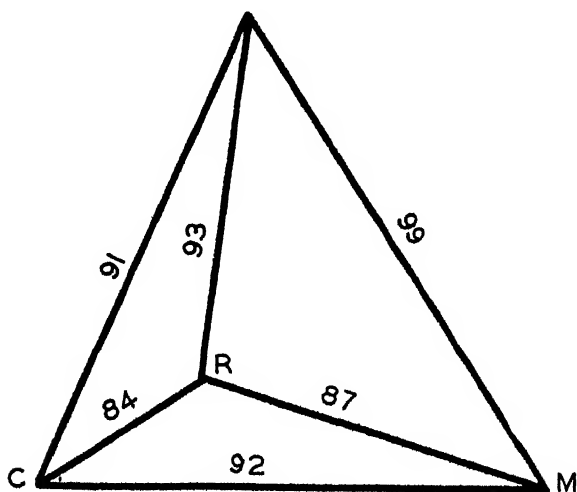


FIGURE 3. A diagram to show the relative distances of four families of Orthoptera from each other. The data are tentative inasmuch as the families have not yet had really adequate testing. The families concerned are Blattidae (*R*), Mantidae (*M*), Gryllidae (*C*), and Acrididae (*G*). The figure requires three dimensions for adequate representation, hence values contiguous to the Blattidae locus are not in proportion with the remainder of the figure. A value of 99 represents the maximum measurable distance between any two families.

represented in Figure 3 inasmuch as no antiserum was produced against this family and reciprocal relationships could not be established. It should be pointed out that the values given are not absolute but only relative. A summary of the relationships of these Orthoptera as determined by the comparison of extracts made from whole insects is as follows:

- A. Blattidae—approximately equidistant from the Mantidae and Gryllidae and closer to these families than to the Acrididae.
- B. Mantidae—most closely related to the Blattidae. Approximately equally related to the more distant Gryllidae and Acrididae.
- C. Gryllidae—closer to Blattidae than to Mantidae and Acrididae.
- D. Acrididae—appears to be more closely related to Gryllidae and Blattidae, than to the Mantidae.
- E. Tettigoniidae—most distant from the Mantidae and Blattidae of all the families tested. Shows slightly more relationship to the Acrididae than to the other families.

There is striking correlation between the findings of Crampton (1932) in his taxonomic and phylogenetic studies and the orientation of insect families as discussed above, indicating that there may be some correlation between time of origin and degree of similarity in the antigenic constituents of insects, i.e., their serum proteins and other extractable proteins. Except for the position assigned to the Gryllidae, there is also general agreement between the phylogenetic tree of Walker (1922) and the relative positions occupied by the five families of Orthoptera investigated in this paper. As stated previously, however, systematic serology does not attempt to give a final answer to questions on the phylogenetic origin of organisms, but rather associates animals as they exist today into natural classifications based on their essential biochemical similarities. Where serological evidence is in agreement with a paleologic-taxonomic study which correlates phylogenetic origin and present day classifications, then the biochemical (i.e., serological) evidence becomes additive to the other two and increases the likelihood that such a study presents the true picture of the evolutionary development of the species examined.

### SUMMARY

1. Representatives of five families of the insect order Orthoptera were compared serologically.
2. On the basis of the degree of serological similarity among them the relative positions of these five families are given.

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# THE ACTION OF ELECTRICAL STIMULATION AND OF CERTAIN DRUGS ON CARDIAC NERVES OF THE CRAB, CANCER IRRORATUS

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## INTRODUCTION

The nervous regulation of the decapod crustacean heart has been studied for over a century, yet the nature of the mechanisms involved are still obscure. The nerves concerned are minute, and only in recent years has a picture been obtained of the anatomical relationships of the cardio-regulatory nerves to the heart and its ganglion cells.

Inhibitory cardiac nerves in crayfish were early indicated or demonstrated by several investigators including Dogiel (1876, 1877), Yung (1878), and Plateau (1878, 1880), whose work showed in a general way that these nerves arise from the anterior part of the thoracic ganglionic chain or mass. Dogiel believed that nerves arising anterior to the sternal artery and running to the pericardium and abdominal extensor muscles were inhibitory, but as his only points of electrical stimulation were the pericardium and the thoracic nerve cord between the bases of the second and third leg nerves it does not appear that he traced the inhibitory nerves in detail. In view of frequent misquotations it is well to note that "Dogiel's nerve" was never claimed to run along the sternal artery or actually to enter the heart. Inhibitory cardiac nerves in the crayfish have recently been traced by Wiersma and Novitski (1942).

Definitive information on the course of inhibitory cardiac nerves in crabs rests upon the work of Jolyet and Viallanes (1892, 1893), Conant and Clark (1896), and Bottazzi (1901). Conant and Clark demonstrated most clearly in *Callinectes* that the inhibitory nerves arise as a single pair close to the bases of the recurrent cutaneous nerves, and run with these large nerves anterodorsally from the thoracic ganglion, eventually separating and joining the cardio-accelerator nerves on each side to form the lateral pericardial plexus. The entrance of the inhibitory nerves into the heart was not observed by Conant and Clark, but the observations of Alexandrowicz (1932), Heath (1941), and the writer are in agreement that the cardio-regulatory nerves enter the crab heart as a single dorsolateral pair of delicate strands, containing very few fibers.

Interest in cardio-accelerator nerves at first centered about "Lemoine's nerve," which was described in the crayfish by Lemoine (1868) as arising from the stomatogastric nervous system on the anterior dorsal wall of the stomach. This nerve, which is extremely fine, was reported to run beneath the ophthalmic artery and to break up as it entered the heart. In view of later misquotations, it is well to note

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that Lemoine did not derive his cardiac nerve from the cerebral ganglia, nor did he produce any effects on the heart by stimulation of the "brain." The positive results of stimulation of "Lemoine's nerve" reported by several authors (Lemoine, 1868; Yung, 1878; Plateau, 1880) were possibly a reflex phenomenon. More recent workers, including Jolyet and Viallanes (1892, 1893), Conant and Clark (1896), Wiersma and Novitski (1942), have not found "Lemoine's nerve" to be a cardio-accelerator. Vital staining studies by Alexandrowicz (1932), Heath (1941) and the writer show that this nerve in the crab innervates the anterior valves of the heart, and does not pass to heart ganglion or muscle. Jolyet and Viallanes (1892, 1893), confirmed by Conant and Clark (1896), showed that the cardio-acceleratory nerves in crabs consist of two pairs arising dorsal to the bases of the nerves to the third maxilliped and the first leg. The two acceleratory nerves on each side join the single inhibitory nerve to form the lateral pericardial plexus, from which the acceleratory nerves pass into the heart in the same thin strand which carries the inhibitors.

The cardiac ganglion (intrinsic ganglion) of the crab heart, as in other marine decapods, contains about nine cells and sends its fibers in a complex but regular pattern throughout the heart (Alexandrowicz, 1932). It is innervated by the single pair of nerves carrying inhibitory and excitatory fibers. The exact relationship of each type of fiber to the heart ganglion and heart muscle is not fully understood. Alexandrowicz and others have observed that the fibers entering the heart are of two sizes, the larger of which make synaptic contact with processes of the ganglion cells, while the smaller fibers pass to the muscle. Alexandrowicz suggests that the thicker fibers are inhibitory, the thinner excitatory. However, in their studies of the multiple innervation of crustacean limb muscles, van Harreveld and Wiersma (1939) have found that the inhibitory fiber is thinner than the motor fibers to the same muscle, while Wiersma and Novitski (1942) have shown that the cardio-acceleratory nerves in the crayfish probably act upon the heart ganglion rather than on muscle.

Pharmacological studies have indicated that a cholinergic nervous mechanism is concerned in the initiation of heartbeat in higher crustaceans (Welsh, 1939a, 1939b; Davenport, Loomis, and Opler, 1940; Davenport, 1941, 1942; Prosser, 1942 and others). The isolated decapod heart is stimulated by acetylcholine, an effect augmented by eserine and blocked by atropine. While it is generally agreed that the intrinsic ganglion has a pacemaker function, and that it is stimulated by acetylcholine (Welsh, 1942), attempts to determine whether acetylcholine acts in a muscarine-like or in a nicotine-like fashion on the ganglion or muscle of the isolated heart (Davenport, 1941, 1942) have revealed little concerning the manner of action of the regulatory nerves which reach the heart from the central nervous system.

In pharmacological studies of the isolated heart it has generally not been possible to tell at what sites the drugs used are actually taking effect. For instance, the rate of the isolated crustacean heart is quickened by acetylcholine. This effect is probably due to the action of the drug on the heart ganglion, but the possibility of a direct action on the muscle remains. The sensitivity of the ganglion to acetylcholine does not prove that the neurons of the ganglion are themselves cholinergic. They might be cholinergic, adrenergic, or of some still unknown nature. It is helpful to recall that in vertebrates cholinergic preganglionic fibers may stimulate either

cholinergic or adrenergic postganglionic neurons in the autonomic nervous system. Thus the situation in the isolated crustacean heart is more complex than it may appear at first glance. When a drug is applied to the whole heart, it undoubtedly has some effect on muscle as well as on the ganglion, and if we admit the possibility of its stimulating the remnants of regulatory nerve still within the heart, we see further complications, not only because we may not be able to identify the effects of the regulatory nerves in the total response, but also because the simultaneous stimulation of these opposing nerves could produce conflicting effects. Since the completion of the work reported here, Wiersma and Novitski (1942) have shown that perfused acetylcholine and excitatory nerve stimulation produce similar effects in the crayfish heart, and that eserine augments the effect of nerve stimulation upon heart rate. These observations may be considered as excellent evidence for the cholinergic nature of the cardio-accelerator nerves and for the termination of these nerves upon the ganglion cells rather than on muscle. However, it is still not known at what point in the heart the inhibitory nerves act. Previous studies by the writer on nervous inhibition of the heart of *Panulirus* (Smith, 1940-41) led to the conclusion that the cardiac ganglion was possibly cholinergic, but that the inhibitory mechanism was not of the "muscarine-like" cholinergic type.<sup>2</sup>

In view of the scarcity of direct evidence on the nature of the regulatory heart nerves it was felt that further study of the effects of certain drugs on the action of these nerves in a semi-intact preparation might yield information regarding their nature and mode of action. It was originally hoped that this work could include an electrical study of the heart ganglion when the heart was being inhibited or excited by nervous action, in order to clarify the role of the heart ganglion in nervous control, but, unfortunately, exigencies of 1942 did not permit electrical studies to be made.

Throughout this work I received constant encouragement and much helpful criticism from Dr. John H. Welsh, to whom I wish to express my gratitude.

#### MATERIAL AND METHODS

The eastern rock crab, *Cancer irroratus*, was found to be of suitable size and hardiness for winter laboratory use if maintained under cold conditions. By suitable cannulation, the heart was perfused *in situ* with physiological solutions or drugs. Heart action was recorded kymographically, while stimulation was applied to the inhibitory and to the excitatory nerves by means of two sets of mechanically manipulated electrodes which were left in place upon the nerves through the course of an experiment. Exposure of the thoracic nerve mass from beneath gave access to these nerves close to their point of origin, at a considerable distance from the heart. Stimulation was supplied either by a pair of Harvard inductoria, or by a thyatron stimulator (modified after Delaunois, 1939) giving repetitive shocks at controllable frequencies and voltages.

Perfusion fluid was made up following Cole's (1940) analysis of the blood of *Cancer borealis*. (An analysis of the blood of *C. irroratus*, generously carried out by Dr. Cole, showed that the composition of the blood of this species is similar to that of *C. borealis*.)

<sup>2</sup> The statement in the original report (Smith, 1940-41) that, "the inhibitory mechanism is not of the cholinergic type," was not justified by experimental results, and should be in the less sweeping form, "not of the muscarine-like cholinergic type."

NaCl	0.506 M	1000 parts			
KCl	0.506 M	26.1 parts	MgCl <sub>2</sub>	0.506 M	8.7 parts
CaCl <sub>2</sub>	0.506 M	26.1 parts	MgSO <sub>4</sub>	0.506 M	39.1 parts

To each liter of solution was added 17.6 cc. of 0.5 M boric acid and 0.96 cc. of 0.5 M NaOH as a buffer.

The crab heart perfused *in situ* exhibits a steady beat which may be slow or rapid, depending upon the temperature and the conditions of the dissection and perfusion. For best results, all the supporting ligaments of the heart should be intact, although a fairly good beat may often be obtained when the posterodorsal ligament has been severed. A low temperature (10° – 15° C.) is much more favorable than room temperature at any season. The pressure within the heart, which depends upon the rate of perfusion as well as upon the integrity of the heart and its valves, largely controls the amplitude and rate of beat. In diastole the heart is stretched horizontally by its elastic supporting ligaments until it presents a broad, flat or slightly depressed dorsal surface. In systole, the internal pressure causes the dorsal surface to bulge upwards as the heart passes from a flat to a more rounded cross-section, while at the same time, the rear wall of the heart bulges posteriorly. This latter motion was recorded in the tracings as a sharp upward deflection of the writing lever.

With insufficient internal pressure, the heartbeat is weak and irregular, or may even cease. Too great pressure causes a marked increase in amplitude and rate of beat. In this work, the perfusion rate was adjusted until the heart was beating steadily, and not showing more than slight passive swelling between beats. Sudden increases in the rate of perfusion produce an increase in the amplitude and rate of beat strikingly similar to that caused by the excitatory nerves. That this is not a reflex phenomenon involving these nerves can be shown by its continuance after complete removal of the central nervous system. On the other hand, a decrease in the pressure exerted within the heart by the perfusion fluid may cause slowing or cessation of beat. These results of internal pressure changes will be found helpful in explaining certain after-effects of nervous excitation and inhibition.

## EFFECTS OF FARADIC STIMULATION OF CARDIO-REGULATORY NERVES

### *Nervous inhibition*

The inhibitory nerve to the heart was stimulated close to the basal portion of the recurrent cutaneous nerve, just forward of the ventral thoracic ganglion (see Fig. 1). With the ventral approach to the inhibitory nerves, there is little danger of confusion from accidental stimulation of the excitatory nerves. In this work, stimuli only slightly above threshold have been used, and in properly set up preparations no effects of any spread of current from the point of stimulation have been noted.

With inductorium stimulation, cardiac inhibition is usually complete, there being only a very narrow range in which partial inhibition can be obtained. For this reason, the inductorium is useful mainly to determine the intensity threshold for complete inhibition. The inhibition obtained by these means is an abrupt cessation of heartbeat when the intensity threshold is reached. This persists for ten to sixty seconds if the stimulus is continued, after which "escape" beats occur, leading to restoration of a normal beat. If the stimulus is discontinued while the heart is stopped, the beat is restored immediately, without perceptible inhibitory after-



effect. In a few cases, the first beats after inhibition are of increased amplitude or rate. Observation has shown that during the period of inhibition, the continued inflow of fluid sometimes stretches the heart passively, as indicated by a rise in the traced record. When a stretching of the muscle occurs, the effect at the restoration of beat is the same as that resulting from an increase in perfusion rate, hence the heart responds by an augmentation of beat. In cases where the rate of perfusion is low, or when the heart stopped in diastole still tends to drain naturally, there is no evidence of distention, and as a result, no stimulatory after-effect of inhibition.

### *Nervous excitation*

The two pairs<sup>3</sup> of excitatory nerves arise dorsal to the bases of the nerves to the third maxillipeds and the chelipeds respectively (see Fig. 1). Stimulation of these nerves in the vicinity of the heart is not effective because of interference from the nearby inhibitory fibers, but they are easily stimulated as they leave the thoracic ganglion. Commonly, the electrodes were placed to the rear of either of the large nerves mentioned above, dorsal to whose roots the heart nerves arise. However, because of the fact that the inhibitory nerves arise only slightly anterior to the excitatory, it is advisable to sever the inhibitory nerve on the side where the excitatory nerves are being stimulated. Excitation caused by faradic stimulation does not show a sharp threshold. It develops gradually with increasing intensity of stimulation, and soon reaches a maximum. One cannot well speak of "partial excitation." It would have been desirable to establish a quantitative measure of the amount of excitation produced, but because of the great variation in the character of the response in different hearts no reliable method was devised. According to the condition of the valves after the manipulation attending cannulation, the accelerated heart may literally pump itself dry, or it may retain fluid and increase the internal pressure. With these and possibly other variables operating to modify the picture of nervous augmentation of heartbeat, faradic stimulation of the excitatory nerves may produce an increase either in rate or amplitude, or in both. The after-effect of excitation is likewise variable, but in contrast to inhibition, excitation commonly shows a stimulatory after-effect, which in some cases lasts for several minutes after stimulation has ceased. This is most noticeable when a preparation is fresh.

A factor which may act to conceal a stimulatory after-effect, and which may even result in a depression, is the tendency of some hearts to pump themselves dry when accelerated, leading to temporary cessation of beat following the period of excitatory stimulation. Nevertheless, the number of times that excitation of the heart has resulted in an unquestionable after-effect in these experiments, as well as in those of earlier workers (Bottazzi, 1901; Conant and Clark, 1896), makes it appear that this phenomenon is a characteristic feature of nervous excitation of the crab heart, as a result of faradic stimulation.

That there is an upper limit to the effectiveness of the excitatory nerves is shown by their inability to produce a state of tetanic contraction in the heart, although the heart may readily be tetanized by direct stimulation, while acetylcholine

<sup>3</sup> No excitation of the heart by stimulation of "Lemoine's nerve" has been noted in this work.

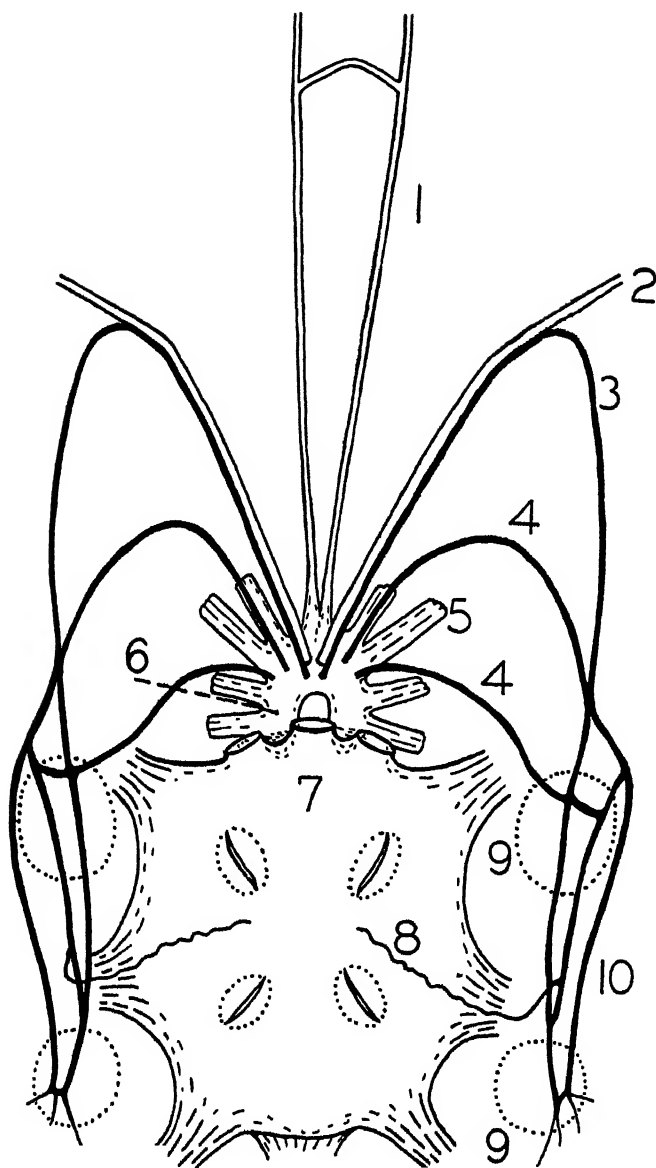


FIGURE 1. Schematic representation of the innervation of the heart of *Cancer* in dorsal aspect, with the thoracic ganglion shown as if moved forward slightly. Approximately to scale, but with cardiac nerves and pericardial plexus drawn with disproportionately heavy lines. (1) Circumoesophageal connective, (2) recurrent cutaneous nerve, (3) inhibitory cardiac nerve, (4) (4) excitatory cardiac nerves, (5) nerve to cheliped, (6) thoracic nerve mass, (7) heart, (8) dorsal nerve entering heart, (9) (9) branchio-cardiac orifices, (10) lateral pericardial plexus.

in high concentrations has been shown to cause an accelerated beat leading to a tetanus (Welsh, 1939a, b; Davenport, 1941).

Such results indicate that the extrinsic excitatory nerves do not directly control the rate of heartbeat, but must act to alter the rate of spontaneous activity in the pacemaker mechanism of the heart. Presumably the effectiveness of these nerves lies in their ability to alter the conditions determining the activity of the cardiac ganglion without being able to force the latter beyond certain limits.

It is commonly stated that the crustacean heart muscle, unlike the vertebrate heart, shows no absolute refractory period. This generalization holds when the heart is stimulated via the excitatory nerves, since cardiac excitation may commence at any phase of the beat, resulting in a well-marked summation. In this respect, the crustacean heart reacts rather more like skeletal muscle than like vertebrate cardiac muscle.

#### EFFECTS OF FREQUENCY OF STIMULATION UPON THE CARDIO-REGULATORY NERVES

In an effort to obtain a clearer picture of the action of the cardio-regulatory nerves, they were stimulated over a wide range of frequencies, employing a thyatron stimulator which delivered repetitive shocks over a range of 10 to 1,800 per second, with voltage adjustable from 0 to 10 volts. This stimulator could be connected at will to either of the two sets of electrodes in use, allowing comparable studies to be made on excitatory and inhibitory nerves in the same preparation over the same period. Both electrodes were fixed in place and undisturbed through an experiment.

Preliminary experiments showed that at very low or high frequencies the voltage required to produce a response was higher than at intermediate frequencies. Accordingly, a series of trials was made to determine the threshold voltage at various frequencies. Frequency-intensity threshold curves were plotted, showing the changes in intensity threshold over a wide range of stimulation frequencies, and the frequency limits above and below which stimulation of the heart nerves produced no effect. The results of a typical experiment are shown in Figure 2.

The greatest difficulty in this method is the problem of recognizing the onset of response in the heart at low or high frequencies outside of the optimum range. Over most of the frequency range, inhibition is total and begins abruptly, so that the threshold is easily determined, but at low frequencies, inhibition may first appear as a slight, gradually increased slowing of the heart, which may or may not cease beating abruptly when a higher voltage is reached. In such cases, two thresholds must be noted, one for partial, and one for total inhibition. In the case of excitation, while the onset of response is sharp over most of the frequency range, there may be a gradual acceleration at low frequencies that is especially hard to detect. It has not been possible to establish any criterion of "partial" excitation, hence the first noticeable increase in rate or amplitude has been taken as the threshold. Obviously, in all tests of this sort it is important to raise the intensity at a uniform rate, ceasing to raise the intensity as soon as results are observed on the kymograph tracing. Further to lessen the subjectivity of observations, tests were carried out in a planned series without stopping to verify individual readings. The series of frequencies were then passed through in the reverse direction in such a way that descending frequency settings alternated with those of the ascending series. No curves were con-

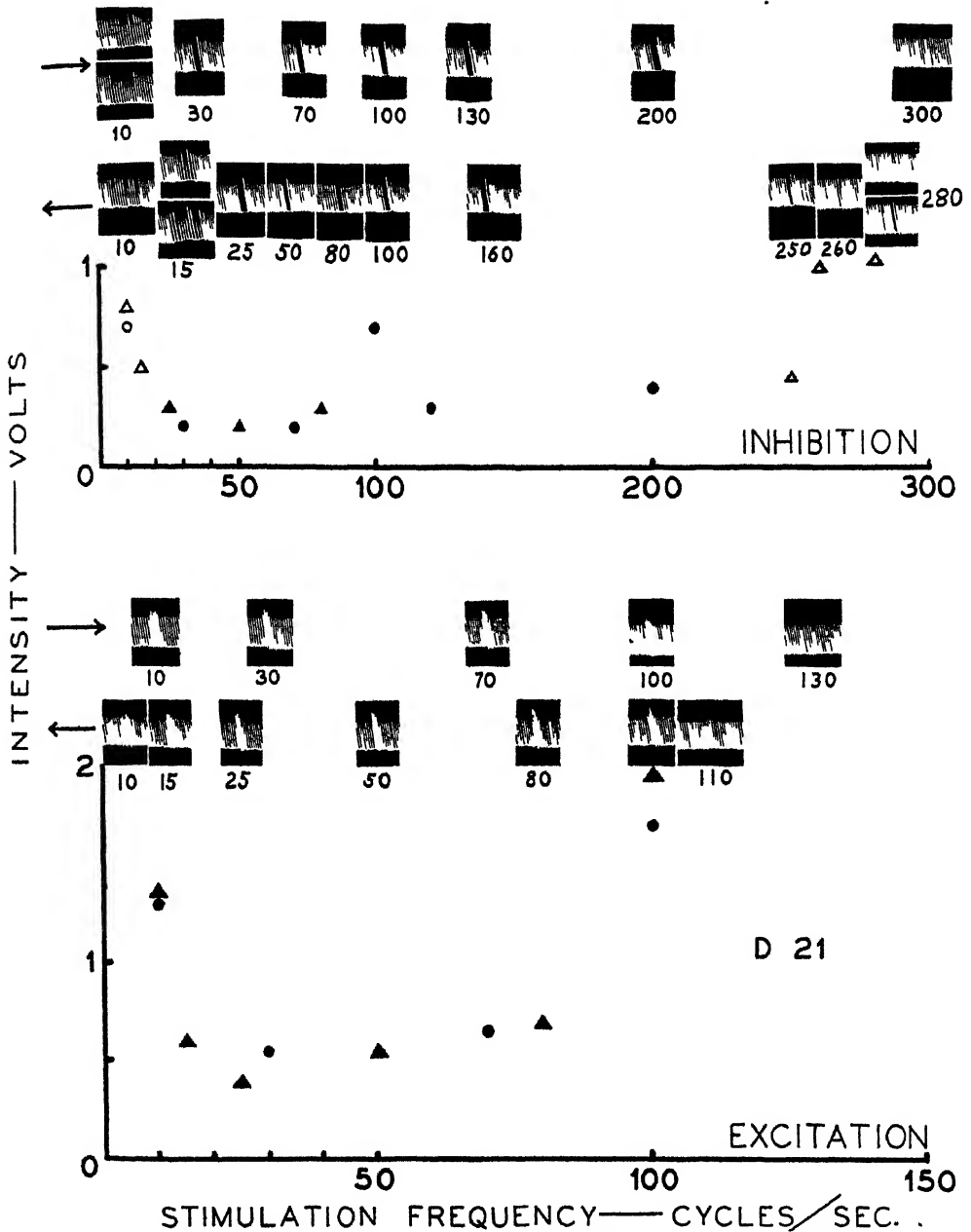


FIGURE 2. Frequency-intensity threshold curves for stimulation of the cardiac inhibitory and excitatory nerves, with tracing of the heart response at each frequency. Circles on the curve represent determinations made on an ascending scale of frequencies; triangles those on a following descending frequency series. Both curves obtained from the same animal (D-21), with tests made alternately on inhibitory and excitatory nerves. Open symbols indicate thresholds for partial inhibition at frequencies where no total inhibition could be obtained

sidered valid if the electrodes had to be moved during the course of the experiment. Certain generalizations may be made regarding the effects of the frequency of stimulation on the cardio-regulatory nerves.

### *The excitatory nerves*

1. The excitatory nerves show a low intensity (voltage) threshold for stimulation over a wide range of frequencies.

2. The lower frequency limit for effective stimulation of the excitatory nerves is lower than that for the inhibitory nerves. At frequencies as low as 10 per second there is often only a very slight rise in the threshold intensity of stimulation.

3. The upper frequency limit of effectiveness of stimulation of the excitatory nerves is variable, usually between 100 and 300 per second, but in some cases ranging well above 300 per second. In such cases it is unlikely that the nerve is actually discharging at such a high rate. The usual upper frequency limits are in agreement with the findings of Bogue and Rosenberg (1936), who report that in the leg nerves of *Maia* a large proportion of the motor fibers respond regularly to stimuli delivered at the rate of 200 per second, but that at 400 per second the fibers cease to respond in phase.

4. In the lower range of stimulation frequencies, higher voltages are required to stimulate the excitatory nerves, and less effect is produced upon the heart than when higher stimulation frequencies are employed. Raising the intensity of low-frequency stimulation does not produce the marked increase in response of the heart that can be produced by raising the frequency. This finding is consistent with the small number of nerve fibers innervating the heart and with the marked facilitation exhibited by crustacean nerves.

### *The inhibitory nerves*

1. There is a frequency range from about 15–20 stimulating shocks per second up to 100 or 200 per second in which inhibition is complete at or near the threshold voltage of inhibitory nerve stimulation.

2. At a lower range of frequency, inhibition becomes only partially effective. In some preparations, stimulation of the inhibitory nerve at 10 per second has no visible effect on the heart, regardless of the stimulus intensity.

3. At the upper range of effective frequencies, stimulation of the inhibitory nerve often causes only partial inhibition. There is so much variation in the upper limit of effectiveness that no significance can be attached to its value in the individual preparation.

### *The effect of aging of the preparation upon the frequency-intensity threshold curves for inhibitory and excitatory nerves*

Since in the experiments with perfused drugs, to be described below, it was necessary to run repeated series over periods of several hours, a number of undrugged preparations were tested repeatedly in order to determine the effects of fatigue. There is exhibited in successive runs a slight raising of the intensity threshold, associated with a narrowing of the frequency range over which stimulation of the heart nerves is effective. This is most marked at the upper end of the

frequency range, where no certain significance can be attached to it. The lower end of the frequency curve shows much less tendency to shift with aging than does the upper. For this reason, in the experiments with drugs, the main attention was directed to that portion of the frequency curve lying below 100 per second.

#### SIMULTANEOUS STIMULATION OF INHIBITORY AND EXCITATORY NERVES

Using two inductoria, excitatory nerves have been stimulated during the course of nervous inhibition. In all cases where equal intensities have been used, inhibition remains dominant, and is not interrupted even when much stronger intensities of stimulation are applied to the excitatory nerve. Also, if an inhibitory nerve is stimulated during a period of nervous excitation, inhibition readily interrupts the excitation. But since a stronger inhibitory stimulus is required to stop a heart already under the influence of the excitatory nerve than is required to stop the unstimulated heart, it would seem that to a certain extent the inhibitory and excitatory nerves are each able to modify the effects produced by the other.

Further work, especially electrical studies of the ganglionic pacemaker within the heart, is needed before it can be said whether or not the "blending" of the actions of inhibitory and excitatory heart nerves means that these opposing nerves affect the same portion of the pacemaker system.

#### EFFECTS OF STRYCHNINE UPON NERVOUS EXCITATION AND INHIBITION OF THE CRAB HEART

A number of tests have been made of the action of strychnine upon the cardiac inhibitory and excitatory nerves. Because strychnine in the concentrations necessary to affect the regulatory nerves has been found to affect the heartbeat adversely, about half of the experiments have been inconclusive. This is especially true in regard to studies on inhibition, since a heart that has stopped beating cannot be further inhibited, even though it can frequently be made to beat by stimulation of the excitatory nerves. The effects of strychnine on the character of the heartbeat generally follow a sequence that is passed through more or less quickly depending upon the concentration of drug applied. There is first a moderate increase in rate and amplitude of beat, giving way to a phase of grouped beats, in which groups of 2-8 beats are separated by short (2-5 second) pauses. With a decrease in the number of beats in a group, a state is reached in which the heart records single spike-like beats at intervals of 10-30 seconds. These beats commonly pass to the form of brief tetani, lasting some 1-4 seconds, which are a very characteristic result of strychnine in the crab. If poisoning is more severe or long continued, the heartbeats may become irregular or cease altogether, although retaining their amplitude to the last. The heart will usually recover if thoroughly washed.

#### *The effects of strychnine upon nervous inhibition*

Twelve experiments were performed, of which three were discarded because the electrodes had to be moved during the run, indicating poor setting or local injury to the nerve, while in three the heart failed before all the necessary tests could be car-

ried out. The remaining six indicate that strychnine, in concentrations of about 1:2500 ( $4 \times 10^{-4}$ ), is able to abolish the effect of the inhibitory nerves after perfusion for 30 to 60 minutes. The loss of response was verified in all cases by decreasing the coil distance of the stimulating inductorium by 5 mm. After a short period of washing, during which the beat usually showed a temporary depression, the inhibitory effect of the nerve was restored to or nearly to the initial value; in all cases the threshold after restoration was lower than the strongest stimulus used to verify the abolition of inhibition. In each of two experiments the abolition of inhibition in strychnine and its restoration after washing were repeated four times.

#### *The effects of strychnine upon nervous excitation*

The records of the effects of strychnine upon the effectiveness of the excitatory nerves to the heart are somewhat more reliable than are those for inhibition, since excitatory effects may frequently be obtained even after the heart has ceased to beat. Strychnine in concentrations of 1:2500 ( $4 \times 10^{-4}$ ) blocks the action of the excitatory heart-nerves, and this effect is fully reversible.

These experiments indicate that the cardiac regulatory nerves can be paralyzed by strychnine, as the somatic motor nerves presumably are, without causing the heart to cease beating. However, the anomalous character of the beat in the strychninized heart indicates that the intrinsic pacemaker neurones are affected considerably, though perhaps to a lesser degree than are the regulators.

### EFFECTS OF ATROPINE UPON NERVOUS INHIBITION AND EXCITATION

Methods of study involving the use of inductorium stimulation, as in the work on the effects of strychnine described above, failed to reveal any effect of atropine on either the excitatory or the inhibitory nerves. Accordingly, confirmatory studies were made by the method of variable-frequency stimulation. Frequency-intensity threshold curves have been determined before and during perfusion with atropine, as well as after a period of washing.

#### *The effects of atropine upon nervous inhibition*

Atropine sulphate was perfused in concentrations of  $10^{-5}$  and  $10^{-4}$  for periods of 15–30 minutes before determining the frequency-intensity curves for the inhibitory nerves while the heart was still being perfused with the drug. The application of the drug followed, of course, the determination of such a curve while the heart was bathed with perfusion fluid. The cardio-inhibitory nerves of *Cancer* are not blocked by atropine, but are actually slightly augmented in their effects.

#### *The effects of atropine upon nervous excitation*

Atropine sulphate was perfused in concentrations of  $10^{-5}$  to  $10^{-4}$  for 15–30 minutes, as in the preceding experiments. The frequency-intensity threshold curves indicate that atropine in these concentrations has no significant effect upon the cardiac excitatory nerves. That a sufficient concentration of atropine ( $10^{-4}$ ) was used is indicated by the fact that Welsh (1939b) found that atropine  $10^{-5}$

would largely block the effects of acetylcholine on the isolated heart of *Panulirus*, while atropine  $10^{-4}$  is effective in this respect on the heart of *Astacus* (Davenport, Loomis, and Opler, 1940) and atropine  $5 \times 10^{-5}$  on the isolated heart of *Cancer magister* (Davenport, 1941).

#### EFFECTS OF NICOTINE UPON NERVOUS INHIBITION AND EXCITATION

In view of the failure to block nervous inhibition and excitation with atropine, it was of interest to see how nicotine might affect these processes, since certain effects of acetylcholine ("nicotine-like" effects) are not abolished by atropine. A number of experiments were performed, using faradic stimulation. The results have been checked by variable-frequency stimulation. Nicotine, when first applied, has a strong augmenting effect on amplitude and rate of beat. The stimulatory effects wear off after a time, and, if the concentration of nicotine is high, depression of heartbeat ensues. Irregularity and grouping of beats are common in the early stages of nicotine depression; later stages are marked by infrequent beats or by an abnormal condition of intermittent tetanus. This last type of beat may be the result of a direct response of the heart muscle to stretching, as it could be seen in the records that the heart swelled noticeably between "beats." The crab heart adapts to nicotine, hence successive increases in the strength of drug applied produce relatively small effects.

The return of the heart to perfusion fluid after being in a strong nicotine solution frequently results in a temporary cessation of beat, following which the beat may be greatly accelerated as the nicotine concentration falls from a depressant to an excitatory level. During the period of complete stoppage, the excitatory nerve is able to function, an indication that this nerve is more resistant to the paralyzing effects of nicotine than is the cardiac ganglion.

#### *The effects of nicotine upon nervous inhibition*

In no case has inhibition been abolished by nicotine in concentrations of 1:10,000 to 1:1,000. Hearts responding to freshly applied nicotine by a marked frequency increase can be inhibited as readily as the normal heart, or as hearts which have been greatly depressed by strong concentrations of nicotine. The action of the inhibitory nerves can be studied only as long as the heart continues to beat, hence the effect of dosages of nicotine of strengths greater than 1:1,000 could not be determined.

#### *The effects of nicotine upon nervous excitation*

Nicotine in strengths of 1:1,000,000 to 1:500 has been tested, with inductorium stimulation of the excitatory nerves. The results fall into three classes, depending upon the strength of drug used and its effect on the rate of heartbeat. Nicotine in the lower concentrations ( $10^{-6}$ ), or when first applied, has a marked positive chronotropic effect which may persist for some time. In certain experiments, stimulation of the excitatory nerve during this period of excitation has shown an augmentation of the effectiveness of the nerve as compared to its effectiveness upon the undrugged heart.

In intermediate concentrations of nicotine ( $10^{-5}$ ), or in concentrations applied for a length of time sufficient to depress the heartbeat, nervous excitation is not



impaired. This fact indicates that the cardiac ganglion is more sensitive to nicotine depression than are the excitatory nerves. The complete stoppage of heartbeat that may follow the return of a heart to perfusion fluid after being in nicotine supports this view, since the excitatory nerves may stimulate a heart that has ceased its spontaneous beat under such conditions. In this situation the pacemaker ganglion has evidently lost its capacity for spontaneous discharge, although it must still be able to transmit impulses to the heart muscle via the neuromuscular junctions when properly stimulated. In considering the effects of nicotine, it must be kept in mind that the drug may not only block transmission at the nerve endings of the ganglion and extrinsic nerves, but may act on the ganglion cell bodies themselves, causing a loss of their rhythmical activity.

In high concentrations of nicotine the effects vary, depending upon whether the heart gives a slow beat of normal form or whether it passes into a state of irregular intermittent tetani. In the former case, stimulation of the excitatory nerve may still be able to rouse the heart to more rapid beating, but in the latter case the excitatory nerve appears to be ineffective.

Frequency-intensity threshold curves for the excitatory nerves showed no significant changes after perfusion of hearts with nicotine  $10^{-6}$  or  $10^{-5}$ . Higher concentrations of nicotine could not satisfactorily be tested in this way, since the hearts generally failed during the lengthy process of determining the curve. Thus even though there is evidence that certain concentrations of nicotine enhance the response to excitatory nerve stimulation, it has not been possible to show any increased effectiveness of lower rates of excitatory nerve stimulation.

The augmentation of excitatory nerve effectiveness by low concentrations of nicotine may perhaps be comparable to the action of nicotine on vertebrate autonomic ganglia, where low concentrations of the drug augment the effect of pre-ganglionic stimulation. In high concentrations, nicotine blocks the passage of impulses at vertebrate autonomic ganglia. In the crab heart nervous excitation has been blocked by nicotine in high concentrations, but only in cases where the heartbeat is of abnormal form. Is this failure of excitation due to a blocking of the excitatory nerve, or is it due to overstimulation of the heart ganglion? The abnormal form of the heartbeat suggests that the function of the heart ganglion has been impaired. If we accept this explanation, we cannot say whether or not transmission at the endings of the excitatory nerves is being blocked.

To sum up the results of this study of the effects of nicotine on the cardio-excitatory nerves, low concentrations of nicotine augment the action of these nerves, and high concentrations appear to block their effects. But, because of the likelihood that the heart ganglion is at least as sensitive to nicotine as are the excitatory nerves, it is not possible to say whether the block occurs at the endings of the excitatory nerves themselves or in the heart ganglion. The evidence so far obtained points to the presence of a "nicotine-like" cholinergic mechanism involved in nervous excitation of the crab heart, but it does not yet allow us to decide between the cardio-excitatory nerves and the intrinsic heart ganglion as possible sites of this mechanism. Perhaps both possibilities are true, since any effects of nicotine observed could be explained on such a basis. The methods employed in this work do not enable us to distinguish between nicotine-block of the cardiac ganglion alone and simultaneous nicotine-block of the excitatory nerve and the heart ganglion.

## EFFECTS OF ESERINE UPON NERVOUS EXCITATION AND INHIBITION

In the crabs used in the present work, eserine (physostigmine sulphate Merck) in concentrations of  $10^{-6}$  or  $10^{-5}$  was found to potentiate markedly the effects of low concentrations of acetylcholine. Hence such concentrations of eserine were considered sufficient to inactivate a significant amount of the cholinesterase in the heart when perfused for 15 minutes or more. Such low concentrations of the drug do not, by themselves, visibly affect the heartbeat.

*The effects of eserine on nervous inhibition*

Eserine in concentrations of  $10^{-6}$  to  $4 \times 10^{-5}$  did not augment the action of the cardio-inhibitory nerves when tested by inductorium stimulation, thus agreeing with results previously obtained on *Panulirus* (Smith, 1940-41), nor could any effect of eserine upon frequency-intensity threshold curves be detected.

*The effects of eserine on nervous excitation*

Since the effects of nicotine on cardio-excitation have made it appear at least possible that the excitatory nerves are cholinergic, the action of eserine on the effectiveness of these nerves is of importance. However, no success was obtained in producing an augmentation of excitation by the application of eserine. Inductorium stimulation did not produce detectable results, while frequency-intensity threshold curves show little more. Eserine either has no effect, or it actually makes cardio-excitation more difficult to obtain. It should be recalled that the concentration of eserine used was sufficient to augment the action of acetylcholine on the crab heart, but that it did not by itself cause any stimulation of the heart. Under such conditions, Wiersma and Novitski (1942) have found eserine to augment the effects of nervous excitation of the crayfish heart, as determined by a study of heart rate.

## EFFECTS OF ACETYLCHOLINE PLUS ESERINE UPON NERVOUS EXCITATION AND INHIBITION

It has been found that a low concentration of acetylcholine chloride ( $10^{-7}$ ) has a long-continued stimulatory action upon the heart of *Cancer* if it is potentiated with eserine  $10^{-5}$ . Neither drug has any marked or persistent effect if perfused alone. Such a mixture of drugs has been used to determine the effects of acetylcholine upon the action of the regulatory nerves. But as in the case of eserine alone, "protected" acetylcholine in low, stimulatory concentrations has no consistent or significant effect either upon the inhibitory nerves or upon the excitatory nerves. Because higher concentrations of acetylcholine gave effects too strong to be maintained over the period necessary for the type of observations made in this study, they were not used.

## DISCUSSION

The lack of effect of eserine and acetylcholine upon the excitatory nerves would seem to speak against the possibility that these nerves are cholinergic. Yet there are indications that the failure to detect eserine potentiation of the effectiveness of these nerves is not conclusive evidence of their non-cholinergic nature. Welsh (1940-41) has reported that while eserine augments the effects of low concentra-

tions of acetylcholine on the isolated lobster heart, it fails to potentiate, or even lessens the effects of acetylcholine stronger than  $10^{-7}$ . It is probable that cholinergic nerves release at their terminations acetylcholine in small amounts but in high, sharply localized concentrations; while cholinesterase has been shown to be greatly concentrated at the motor end plates in vertebrate skeletal muscle (Marnay and Nachmansohn, 1938; Nachmansohn, 1939). In the case of vertebrate autonomic ganglia, Brown and Feldberg (1936) have shown that eserine can potentiate the effect of preganglionic stimulation so effectively that the ganglion cells are soon paralyzed by the accumulation of acetylcholine unless submaximal preganglionic volleys and low frequencies of stimulation are employed. If such a situation exists in the crab, it is possible that eserine may allow the accumulation of mediator substance in depressant concentrations. In view of the evidence of Wiersma and Novitski (1942) that eserine augments nervous excitation of the crayfish heart, a restudy of this matter in the crab would be desirable, employing a technique by which heart rate, uncomplicated by amplitude changes, could be observed.

The inability of the excitatory nerves to tetanize the crab heart, although this state may be produced by direct electrical stimulation, acetylcholine, nicotine, etc., raises the question of whether or not the excitatory nerves act in the same way or at the same point as these other agents. Prosser (1943) has shown that the systole of the neurogenic heart of *Limulus* is initiated by synchronous activity in the cardiac ganglion, while tetanus results from a continued asynchronous discharge. During the course of the present work it has been observed that crab hearts depressed by nicotine or strychnine often exhibit a series of very irregular abnormal beats which, during recovery, regroup themselves to normal systoles, apparently by a return of the several cardiac ganglion cells to synchronous activity. The short tetani that may replace the normal systoles under the influence of nicotine or strychnine may, likewise, possibly be caused by asynchronous discharges of ganglionic cells. Direct electrical stimulation, acetylcholine, and other agents capable of tetanizing the neurogenic arthropod heart may do so by throwing the cardiac ganglion into a state of asynchrony, but it would seem that the excitatory nerves are capable of producing only more closely spaced bursts of synchronous ganglionic activity.

Since other evidence so far accumulated supports the view that the heart ganglion is cholinergic, and since the lack of effect of atropine upon normal heart-beat indicates that the ganglion does not exert a "muscarine-like" action on the heart, muscle, we may consider the possibility that the ganglion has a "nicotine-like" effect at the neuromuscular junction. It will be recalled that, in its action on different vertebrate tissues, acetylcholine has a variety of effects, which may be broadly classified into "muscarine-like" effects that can be blocked by atropine, and "nicotine-like" effects that can be blocked by high concentrations of nicotine. These classifications of acetylcholine action apply, strictly, to the reactions of vertebrate tissues, and it is possible that the reactions of invertebrate tissues do not fall into identical categories. Hence, the fact that the crustacean heart ganglion does not appear to exert a "muscarine-like" action upon muscle does not prove by elimination that its effects are fully comparable to the "nicotine-like" action of certain cholinergic nerves in vertebrates. For the present, however, we may consider that the cardiac ganglion transmits impulses to the heart muscle by a cholinergic mechanism having a somewhat "nicotine-like" effect.

If it is true that both the ganglion and the excitatory nerves are of the same pharmacological nature, this fact renders useless our attempts to demonstrate a nicotine block of the excitatory nerves. As pointed out above, the ganglion seems more sensitive to nicotine than are the excitatory nerves. Paralysis of the ganglion removes an essential link in the chain of impulses which pass from regulatory nerve to heart muscle in nervous excitation. With the ganglion out of action, the present methods do not reveal whether or not the excitatory nerves are blocked by high concentrations of nicotine, but the positive evidence so far obtained points to a cholinergic "nicotine-like" action of the cardio-excitatory nerves upon the heart ganglion.

#### SUMMARY

1. The heart of *Cancer irroratus* has been perfused *in situ*, the beat recorded mechanically, and the inhibitory and excitatory nerves stimulated while the heart was perfused with various drugs.
2. Nervous inhibition of the heart induced by stimulation of the inhibitory nerves is usually total with no inhibitory after-effect.
3. Nervous excitation of the heart induced by stimulation of the excitatory nerves frequently shows a stimulatory after-effect.
4. The cardio-inhibitory nerves appear to be more effective than the excitatory nerves, but each type of nerve can modify to some extent the effectiveness of the other.
5. The excitatory nerves are effective when stimulated at frequencies ranging from 200–300 per second down to 10 per second or less.
6. The inhibitory nerves produce total inhibition when stimulated from 15–20 to 100–200 times per second. At frequencies extending above and below this range, partial inhibition may be obtained.
7. Strychnine, in concentrations of 1:2,500, blocks reversibly the action of both excitatory and inhibitory nerves.
8. Atropine is without effect upon excitatory and inhibitory nerves, as well as upon the heart ganglion in concentrations sufficient to abolish the action of applied acetylcholine.
9. Nicotine, in low concentrations, augments the effectiveness of the excitatory nerves as well as of the heart ganglion.
10. Nicotine in high concentrations may block the passage of impulses from cardiac ganglion to muscle; hence it could not be determined with certainty if nicotine blocks the excitatory nerves.
11. Neither eserine alone, nor with acetylcholine in stimulatory concentrations, augmented cardio-inhibition or excitation. It is suggested that the lack of effect of eserine upon the excitatory nerves deserves restudy.
12. It is probable that the excitatory nerves are cholinergic, and exert a somewhat "nicotine-like" effect upon the heart ganglion, which in turn exerts a "nicotine-like" action at the neuromuscular junction.
13. No light has been thrown on the pharmacological nature of the cardio-inhibitory nerves.

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# THE BIOLOGICAL BULLETIN

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## PHYSIOLOGY OF INSECT DIAPAUSE. II. INTERACTION BETWEEN THE PUPAL BRAIN AND PROTHORACIC GLANDS IN THE METAMORPHOSIS OF THE GIANT SILKWORM, PLATYSAMIA CECROPIA

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In the *Cecropia* silkworm the progress of metamorphosis is interrupted as soon as the pupa is formed. There then intervenes a prolonged period of pupal diapause characterized by cessation of growth and differentiation. The mechanism that converts this cellular dormancy of diapause into the intense activity of adult formation was examined in a previous investigation performed on several species of giant silkworms (Williams, 1946b). For these species it was evident that diapause is under the control of the insect's brain. This control consists in a dependency of adult development on the action of a factor arising from the brain; the brain, in turn, is rendered competent to release this factor by exposure to low temperatures. It is the purpose of the present report to describe in greater detail the nature of the activating mechanism.

### MATERIALS AND METHODS

The present communication is based on a total of 282 experiments performed, for the most part, on pupae of the giant silkworm, *Platysamia cecropia*. In a few experiments pupae of related genera were studied, including *Telea polyphemus*, *Callosamia promethea*, and *Samia walkeri*. The management of this array of animals was essentially identical to that described previously (Williams, 1946b).

### DIRECT OR INDIRECT ACTION OF THE BRAIN?

If the brain is removed from a diapausing pupa, the resulting insect never develops further and persists until death in permanent diapause—a matter of two years in some individuals. Yet at any time during this period adult development can be evoked by implanting into the brainless pupa a brain obtained from a previously chilled pupa.

It was a rational assumption that the developmental factor from the implanted brain had some direct action on the host tissues whereby the latter were converted from dormancy to activity. Yet the possibility remained that the brain's action might be indirect rather than direct, in a sense familiar to endocrinologists, as, for

<sup>1</sup> The assistance of the Society of Fellows, Harvard University, and the Lalor Foundation, Wilmington, Delaware, is gratefully acknowledged.

example, in the action of certain of the pituitary hormones on the uterus. Experiments have therefore been performed to test these two possibilities.

To this end techniques have been developed for subdividing individual pupae into fragments. In my experience division of these stout animals by means of ligatures has not met with success. Nor did gross slicing of the pupae into parts, as practiced by Crampton (1899), Hirschler (1903, 1904), and Hachlow (1931), produce viable preparations. A simple, successful technique that was ultimately developed will be described briefly.

#### ISOLATION OF PUPAL FRAGMENTS

The pupa, under continuous carbon dioxide anesthesia (Williams, 1946a), is placed on its side and the abdominal cuticle plus underlying hypodermis incised around the circumference of the abdomen at the level of the tips of the wingflaps. The incision is confined to the thin intersegmental membrane. Further maneuvers are designed to separate the abdomen from the anterior fragment without rupturing the midgut.<sup>2</sup> The intersegmental muscle masses, the heart, and the nerve cord are, in turn, cut through. The fat body and hindgut are then transected and the attachments of tracheae swept away from the walls of the midgut. The midgut plus nearly all of the Malpighian tubules may now be placed in the anterior fragment. Or by further dissection the midgut may be removed from the anterior fragment and discarded.

The cut surface of each of the two fragments is then sealed by melted paraffin to a circular, plastic cover slip which is provided with a centrally placed hole. Through the hole insect Ringer's is added to displace all air and the hole is finally plugged with melted paraffin.

It may be noted that these isolated abdomens (Fig. 3) consisted, in reality, of the terminal six abdominal segments, the anterior four abdominal segments being carried away with the anterior end (Fig. 1). Transections at other levels were occasionally accomplished, but the one described proved to be most favorable. In

<sup>2</sup> Rupture of the midgut floods the body cavity with a dark green fluid that contains a good deal of particulate matter and shows a broad, dense absorption band centering at 670 m $\mu$ . The fluid is apparently non-toxic, but the particulate matter is frequently drawn into the cut end of the heart. Within the heart it then acts as embolus to occlude the aorta at its narrowest portion just behind the brain. Such animals usually fail to survive.

#### EXPLANATION OF PLATE I

##### Approximately Life Size

FIGURE 1. Anterior fragment of a brainless, diapausing pupa.

FIGURE 2. Fragment in Figure 1, after adult formation. Development was evoked by implanting a previously chilled brain.

FIGURE 3. Posterior fragment of a diapausing pupa.

FIGURE 4. Fragment in Figure 3, after adult formation. Development was evoked by implanting a previously chilled brain plus two pairs of diapausing prothoracic glands.

FIGURE 5. Posterior fragment of a diapausing pupa, showing critical level of section that includes the meso- and metathorax.

FIGURE 6. Fragment in Figure 5, after adult formation. Development was evoked by implanting a previously chilled brain. Note the complete development of those parts of the antennae, legs, and wings that were present in the fragment.

PLATE I



1



2



3



4



5



6



certain instances abdomens were transected a second time to yield viable pairs of abdominal segments sandwiched between plastic slips.

The inner surface of the plastic slips is rapidly coated by a thin, transparent tissue formed by anastomoses developed between blood cells and, within about two weeks, by the outgrowth of epithelium and of tracheoles. At any time, however, one can operate inside the insect fragment by removing the paraffin plug from the centrally placed hole.

Of the entire series of isolated pupal parts, approximately two-thirds died within the first ten days after preparation. Death was invariably preceded by a darkening of the blood, a reaction which in itself seems to be toxic. The remaining preparations survived for considerable lengths of time, as indicated by the beating of the heart and by spontaneous movements of the abdominal segments. Isolated abdomens remained alive at 25°C. for up to eight months. Anterior ends survived for not more than two or three months, however, unless Ringer's solution was occasionally added to compensate for loss of water by evaporation.

#### BRAIN IMPLANTATIONS INTO PUPAL FRAGMENTS

Brainless diapausing pupae were transected at the level of the wing-tips to obtain ten pairs of viable anterior and posterior halves. Into each of these subdivisions a chilled *Cecropia* brain was then implanted (Fig. 1 and 3). Each anterior fragment proceeded to develop normally into the corresponding anterior end of a lively adult moth, the cut surface being closed by scaleless, regenerate, "chitinized" epithelium (Fig. 2). The isolated abdomens, to the contrary, remained undeveloped after brain implantation, although they continued to live for an average of three months.

With slight variations in technique these findings have been confirmed during the past three years on a total of 60 additional viable preparations. As many as six chilled brains have been implanted into a single abdomen without inducing development, even though some of these abdomens survived as long as eight months thereafter.

#### EXPLANATION OF PLATE II

##### Approximately Life Size

FIGURE 7. Posterior fragment of a diapausing pupa joined directly to the tip of a brainless, diapausing pupa.

FIGURE 8. Preparation in Figure 7, after adult formation of host and graft. Development was evoked by implanting a previously chilled brain into the host. Note the regenerate tissue passing through the hole in the plastic slip to connect the two parts.

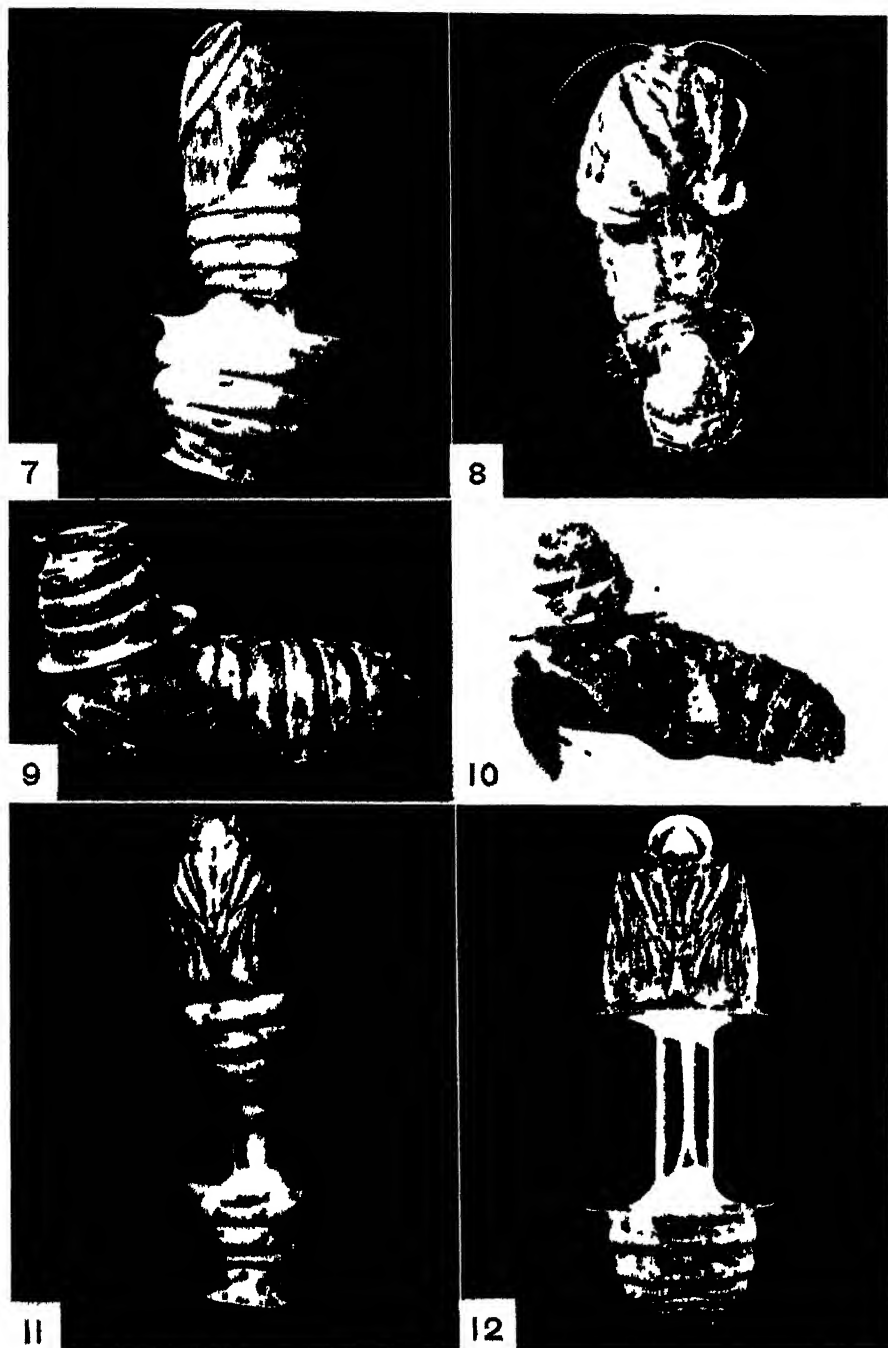
FIGURE 9. Posterior fragment of a diapausing pupa joined directly to the thoracic tergum of a brainless, diapausing pupa.

FIGURE 10. Preparation in Figure 9, after adult formation of host and graft. Development was evoked by implanting a previously chilled brain into the tip of the graft.

FIGURE 11. Posterior fragment of a diapausing pupa joined to a brainless, diapausing pupa by means of a short, square, plastic tube. Note the tube of regenerate tissue traversing the plastic tube's lumen. In order to follow the onset and progress of development, plastic windows have been placed at each end of the preparation.

FIGURE 12. Posterior fragment of a diapausing pupa joined to an anterior fragment of a brainless, diapausing pupa by means of a long, plastic tube. Note the tube of regenerate tissue within the plastic tube.

PLATE II



It is therefore apparent that anterior and posterior fragments respond differently to brain implantation. This difference in response might be explained if the anterior fragment possessed a second developmental center that was lacking in the posterior fragment. To test this possibility the level of transection was varied.

#### TRANSECTIONS OF PUPAE AT VARIOUS LEVELS

Brainless, diapausing pupae were transected at various levels and the developmental capacity of the posterior fragments tested by implanting a chilled brain. The results of these experiments confirmed the findings of previous investigators that a "differentiation center" was present in the region of the thorax of lepidopterous pupae (Hachlow, 1931; Bodenstein, 1938; Bounhiol, 1938). Thus, abdomens in continuity with a pupal thorax invariably developed when a chilled brain was implanted. In contrast, similar treatment failed to evoke development of abdomens separated from the entire thorax. The critical level seemed to lie in the region of the mesothorax, for, after transection at this point, brain implantation induced development only after a long latent period of about six months (Fig. 5 and 6).

#### GRAFTING EXPERIMENTS ON ISOLATED ABDOMENS

The importance of this anterior differentiation center was further revealed in experiments in which isolated abdomens were grafted to brainless, diapausing pupae (Fig. 7, 9, and 11) or to the anterior fragment of brainless, diapausing pupae (Fig. 12). In such combinations, the host and the graft grew together to establish tissue continuity, but did not develop further. This continuity was developed even when plastic tubes, as long as 3 cm., were interposed between the two parts (Fig. 11 and 12).

Now, when a brain from a chilled *Cecropia* pupa was implanted into either host or graft, diapause was terminated and the entire preparation developed into the fully formed, corresponding parts of the adult (Fig. 8 and 10).

#### IMPLANTATION OF PROTHORACIC GLANDS

The evidence, up to this point, indicates that adult development after pupal diapause requires a factor from the brain plus some additional factor from the thorax. An extensive series of experiments was then performed in which a chilled brain plus various thoracic organs were implanted into isolated abdomens.

This search was vastly aided by the publication of Fukuda's paper in 1941. In this communication Fukuda describes an endocrine activity of the "prothoracic glands" in evoking development of ligatured pupal abdomens of the commercial silkworm, *Bombyx mori*.

The great significance of the prothoracic glands was confirmed. Thus, isolated abdomens of diapausing *Cecropia* pupae developed readily when provided with a chilled brain plus two pairs of prothoracic glands.<sup>3</sup> The pupal cuticle became crisp

<sup>3</sup> Due to the numerous ramifications of the pupal prothoracic glands in the pro- and mesothorax, it was usually impossible to remove these organs in their entirety. For this reason two pairs were usually implanted into each isolated abdomen. This proved to be an important detail, for a single pair of the incomplete glands did not generally suffice to produce development after brain implantation. For a description of the prothoracic glands in *Platysamia cecropia* see Williams, 1948.

and was delaminated and the abdomens appeared as the lively, corresponding segments of the adult, the cut surface being closed by scaleless, regenerate, "chitinized" epithelium (Fig. 4). Development was complete externally and internally and, in the case of female abdomens, the eggs were matured. Similarly, abdomens isolated from chilled pupae after return to room temperature required both a chilled brain and prothoracic glands for the initiation of adult development.

In these experiments it was found that the prothoracic glands as well as the brain show a lack of species—or genus—specificity, for both of these organs remain effective when interchanged between *Platysania cecropia* and *Teia polyphemus*.

In contrast to the results of Fukuda (1941), who studied a species without pupal diapause, no development of isolated abdomens occurred when prothoracic glands were implanted in the absence of a chilled brain.

#### FURTHER EXPERIMENTS ON ISOLATED PUPAL ABDOMENS

From a series of six isolated abdomens of diapausing *Cecropia* pupae there were further removed the gonads, the entire digestive tract, the Malpighian tubules (with the exception of a few loose fragments), and the entire central nervous system, including the residual chain of five ganglia and connectives. Three of these preparations survived, the heart continuing to beat. After the implantation of a chilled brain plus prothoracic glands, two of the abdomens developed into flaccid, but fully mature adult abdomens.

#### DISCUSSION

It is evident from these experiments that the termination of pupal diapause requires a minimum of two factors, one from the brain and the other from the prothoracic glands. The easiest way to render these organs functional is to expose the diapausing pupa to low temperatures and then return it to room temperature. As has been shown previously, the effectiveness of chilling in terminating dormancy can be explained in terms of an action of low temperature on the brain, whereby the latter is made competent to release its developmental factor (Williams, 1946b). Manifestly, the prothoracic glands do not require similar exposure to cold, for they are promptly activated when a chilled brain is implanted into a diapausing pupa.

From these observations it may be concluded that the brain exerts a controlling action on the prothoracic glands. Subsequent experiments have confirmed this hypothesis consistently. Thus to induce the development of isolated pupal abdomens, it is necessary that the implanted brain be obtained from a previously chilled pupa. This is not the case in regard to the implants of prothoracic glands, for these organs are equally effective when obtained from diapausing pupae. The functional failure of the prothoracic glands at the outset of diapause seems therefore to result from a primary failure of the brain in releasing its factor.

The mechanism that terminates diapause must ultimately supply the dormant tissues with something necessary for cellular growth and differentiation. In respect to this process the experimental results may be interpreted from two points of view. On the one hand, the brain factor may be conceived as having sole action on the prothoracic glands and the prothoracic gland factor, in turn, as having

specific effect on the tissues of the body in general. On the other hand, the tissues may require interaction with both factors, the first factor serving to condition the tissues for reaction with the second.

Although the evidence at hand does not suffice to exclude either interpretation,<sup>4</sup> it seems likely that the factor from the prothoracic glands has ultimate action on the dormant tissues to convert them to activity. Thus, we have noted previously that adult formation becomes independent of brain action as soon as the earliest

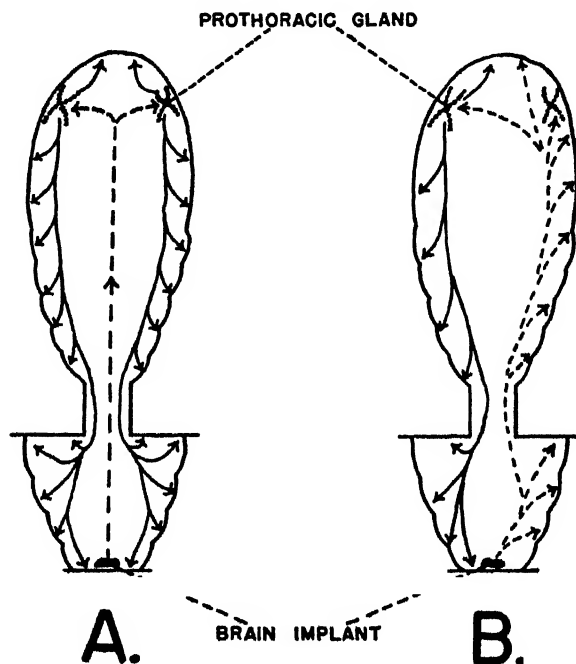


FIGURE 13. Diagrammatic interpretation of experimental results when a chilled brain is implanted into the tip of the graft in preparations such as Figure 11. Development begins first in the anterior end of the pupa, containing the prothoracic glands. For explanation, see text.

sign of adult development is evident (Williams, 1946b). However, for some days thereafter, the animal continues to require further function of its prothoracic glands, for if the abdomen is isolated during this period, it ceases to develop. When tested in this fashion, adult development shows a dependency on the prothoracic glands until a critical stage, signalled by the initiation of eye pigmentation.

Further evidence favoring the view that the factor from prothoracic glands has ultimate action on the tissues has been derived from the experiments, described above, in which isolated abdomens were grafted to brainless, diapausing pupae (Fig. 7 and 9). When development was evoked by implanting a chilled brain *into the tip of the graft*, the brainless pupa, containing the prothoracic glands, was

<sup>4</sup> Since this paper went to press, proof has been obtained of the validity of the first interpretation (see Fig. 13a).

observed to initiate development one day in advance of the abdomen, containing the brain. This difference in time can be magnified to three days or longer by interposing a plastic tube between host and graft (Fig. 11). In such preparations the final organ to initiate development was the imaginal disc of the graft's genitalia, notwithstanding the fact that this lay alongside the implanted brain that had touched off the whole process.

In terms of this type of preparation we may finally summarize our present information diagrammatically, as indicated in Figure 13. In Figure 13*a*, the brain factor is viewed as activating the prothoracic glands and the prothoracic glands as activating the tissues. In Figure 13*b*, the brain factor is conceived to act on all the bodily tissues to prepare them for final reaction with the factor from the prothoracic glands. In either case, the brain is the organ of primary control, but this control, at least in part, is exercised by an indirect mechanism.

### SUMMARY

1. The mechanism that initiates adult development after pupal diapause has been studied in a total of 282 experiments, supplementary to those reported previously.

2. Brainless, diapausing pupae were divided transversely and the developmental capacities of anterior and posterior fragments tested and compared.

3. Implantation of a previously chilled brain sufficed to terminate the dormancy of anterior fragments.

4. Isolated abdomens, to the contrary, remained undeveloped after brain implantation. Yet such abdomens, even without implantation, could be induced to develop by grafting them to developing anterior fragments. Manifestly, the abdomens required for development an additional factor normally produced in the anterior end of the pupa.

5. By transections at various levels the source of this additional factor was found to be the thorax.

6. A testing of various thoracic organs revealed the effectiveness of the "prothoracic glands." Thus, implantation of a chilled brain plus prothoracic glands induced the complete adult development of isolated abdomens. In this effect the prothoracic glands as well as the brain showed a lack of species—or genus—specificity.

7. The termination of diapause requires in these species the action of a minimum of two factors, one arising from the brain and the other from the prothoracic glands. The brain factor is necessary for the activation of the prothoracic glands.

8. The factor from the prothoracic glands, most probably, has ultimate action on the tissues in terminating diapause.

9. The brain is the organ of primary control over diapause in the species studied, but this control, at least in part, is exercised by an indirect mechanism.

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# A COMPARATIVE STUDY OF THE LIPIDS IN SOME MARINE ANNELIDS

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## INTRODUCTION

In the past, studies on the metabolism of lipids have been confined chiefly to observations made on homoiothermal vertebrates. Very little work has been done on the problem in the invertebrates.

Terroine and co-workers (1930) found that the phospholipids in various cold-blooded animals contain very low amounts of fatty acid, that the iodine number of the fatty acids is not constant, and that the iodine number of the phospholipid fatty acids is lower than that of the stored fat but higher than that in similar tissues of homoiothermes.

Timon-David (1930) found that the larvae of 24 insects contained 0.94–28 per cent fat and that linolenic and oleic acids were widely distributed.

Slifer (1932) found that the iodine number of the fatty acids in the eggs of seven species of grasshopper varies from 128–167. She found also that the temperature for complete fusion of the fatty acids depends on the environmental temperature: fats from eggs so laid as to hatch in spring had a melting point decidedly lower than fats from eggs so laid as to hatch in the summer.

Salisbury and Anderson (1939) found that the lipids in *Cysticercus fasciolaris* consist of phospholipids, cholesterol, cerebrosides, and a small amount of neutral fat; that the phospholipids make up about 30 per cent of the total lipids; and that the fatty acids contain saturated and unsaturated forms in the ratio of 1:1.

Wilber (1947) showed that starvation decreases the total lipid in the entire worm *Phascolosoma*, causes little change in the muscle lipids, and decreases markedly the fatty acid and phospholipid content of the coelomic fluid, but does not change the cholesterol content of the latter.

It is evident that information concerning lipid content and metabolism in the invertebrates is quite meager. A detailed study of the problem seems justified. The present report is one in a series dealing with the amounts and use of lipids in invertebrate tissues.

## MATERIAL AND METHODS

Marine annelids were chosen for the investigation because they are obtainable readily and are easy to handle. The following species were studied: *Nereis pelagica*, *Amphitrite ornata*, *Arenicola marina*, *Phascolosoma gouldii*, *Lepidonotus squamatus*, *Glycera americana*, *Chaetopterus variopedatus*. All were procured in the living condition from the Supply Department of The Marine Biological Laboratory during the months of July and August, 1946.



Whole worms or individual tissues were prepared by grinding weighed amounts with sand in a mortar (Bloor, 1929). Lipids were then extracted with boiling alcohol. Some tissues were homogenized in a Waring-blendor instead of grinding with sand. The results from both methods are comparable.

Phospholipids were precipitated with acetone and magnesium chloride and estimated by the oxidation-titration method (Bloor, 1929). Fatty acid and cholesterol were estimated respectively by the oxidation-titration method and by the colorimeter using the acetic anhydride-sulfuric acid reagent (Bloor, 1928). This method measures the total fatty acids in the lipids.

Coelomic fluid was removed from some of the worms and the lipids were extracted and estimated in a similar manner.

From the results obtained, the lipocytic coefficients (cholesterol/fatty acid) and the ratio, cholesterol/phospholipid were calculated for the various tissues (Mayer and Schaeffer, 1913).

## RESULTS

The results are summarized in Table I which shows that there is a wide variation in the absolute values of the various lipid constituents in the different species.

TABLE I

Table showing the amounts of various lipids (values given as per cent of wet weight) in marine annelids and the numerical ratios of the lipids one to another. Values are the mean of ten worms selected at random

Species	Phospho- lipids	Chole- sterol	Fatty acid	Chol. F.A.	Ch. Ph.	Total lipid
<i>Nereis pelagica</i>	0.73	0.35	1.82	0.19	0.48	2.17
<i>Glycera americana</i>	0.82	0.50	2.25	0.22	0.61	2.75
<i>Amphitrite ornata</i>	0.83	0.55	2.60	0.21	0.66	3.15
<i>Arenicola marina</i>	0.35	0.29	0.93	0.31	0.84	1.22
Coelomic fluid*	155	55	877	0.06	0.35	932
<i>Chuetopterus variopedatus</i>	0.33	0.16	0.62	0.26	0.48	0.73
<i>Lepidonotus squamatus</i>	0.23	0.40	3.28	0.12	2.0	3.68

\* Values in mg./100 cc. of coelomic fluid.

The table shows also that tissues with a high content of phospholipid have a high content of cholesterol. A similar relationship is apparent between cholesterol and fatty acid. If the values for fatty acid are plotted against the values for cholesterol, the points are distributed along a straight line.

## DISCUSSION

The results indicate that although there is a wide variation in the absolute values of phospholipid, fatty acid, and cholesterol in the different annelids, there is an apparently consistent ratio of these lipids one to another.

The phospholipids of the annelids studied make up about 37 per cent of the total lipid. This value is close to that obtained (30 per cent) by Salisbury and Anderson (1939) in *Cysticercus fasciolaris*.

It is known that the total lipid in the insect *Sphenarium purpurascens* consists mainly of free fatty acids (Giral, 1946). In the present investigation there is evidence of a predominance of fatty acid over cholesterol and phospholipid in annelid tissue.

The relationship between cholesterol and phospholipids is interesting. The worms with large amounts of cholesterol have a correspondingly large amount of phospholipid. A similar relationship obtains between cholesterol and fatty acid. This may indicate that in the annelids, as in the vertebrates, the ratio of lipids one to another is characteristic of the organ of an animal in a given species (Mayer and Schaeffer, 1913).

The results in general indicate that in the marine annelids, cholesterol is always associated with phospholipids and is in constant relation to them. Cholesterol is, therefore, probably a normal protoplasmic constituent in the annelids as in the vertebrates (Bloor, 1943).

Whether these relationships are constant under different environmental conditions (changes in temperature, salt concentration, or season) is not known at present.

#### SUMMARY

1. The amount of cholesterol, fatty acid, and phospholipid in various marine annelids was estimated.
2. It was found that worms with a high content of cholesterol have also large amounts of phospholipid and fatty acid.

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# THE PROBLEM OF DIFFERENCES OF OFFSPRING IN RECIPROCAL CROSSES OF *DROSOPHILA*<sup>1</sup>

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In general, reciprocal crosses between genetically different groups of organisms yield identical hybrids. In some cases, however, the offspring from reciprocal crosses are different from one another in spite of like nuclear constitution. Such cases are of particular interest, since they bear on the problem of the role of extra-nuclear factors in development and heredity.

In *Drosophila melanogaster* a few examples have been described in which the developmental fate of a zygote depends in part on the genetic constitution of the mother. It seemed possible to obtain further data. A series of reciprocal crosses was performed and the expression of a sensitive indicator phenotype, caused by the mutant "Dichaete," was determined. Several of these crosses seemed to give evidence of a maternal effect. However, further inquiry showed that the significant differences between the outcome of reciprocal crosses were at least partly due to inhomogeneity within each cross, and that no conclusion as to the existence or non-existence of maternal effects was justified. The data and their analysis presented here serve to emphasize the need for detailed statistical study of the range of variability within, as well as between, sets of data under comparison.

## MATERIALS AND BREEDING METHODS

The mutant phenotype "Dichaete" ( $D$ , 3-40.4-41  $\pm$ ) is a very sensitive indicator of external and internal conditions prevalent during the development of *Drosophila melanogaster* (Sturtevant, 1918; Plunkett, 1926). A quantitative measure of such conditions may be obtained by counting the number of dorso-central bristles on the thorax of Dichaete flies. Normal flies usually have four dorsocentrals while Dichaete flies may have from zero to four.

Plunkett has already given some data showing that the mean number of dorsocentrals is not significantly different in the Dichaete offspring from reciprocal crosses between Dichaete and normal flies. The procedure followed in the present study consisted in reciprocal crossing of Dichaete and non-Dichaete flies, the latter of which contained some other mutant genotype. In other words, the expression of Dichaete was measured in populations which not only differed in the maternal or paternal origin of the Dichaete genotype but also in the origin of the egg from a mother either free or in possession of some other mutant genotype. The mutants used in combination with Dichaete were vestigial (2-67.0) Minute (2) (2-12.9  $\pm$ ),

<sup>1</sup> The author wishes to express his appreciation to Dr. Curt Stern for his helpful advice and guidance throughout this investigation. To Dr. D. R. Charles the author wishes to acknowledge in particular his indebtedness for aid in the mathematical treatment of the data throughout.

Minute (3) *w* (3-79.7), Minute 4 (4), and polychaetoid (3-39  $\pm$  4). These mutants hereafter will be referred to as *M-2*, *M-3*, *M-4*, *pyd*, *vg* and *D* (Dichaete). The choice of mutants used was based on the following considerations: (1) *vg*. Hersh and Ward (1932) have reported that *vg/+* heterozygotes possess slightly different wing dimensions, dependent on the direction of the parental cross, vestigial by normal. It was thought that the maternal effect indicated by these findings might express itself also in a modification of the bristle reaction in *D* flies. (2) Minutes. The effect of a Minute constitution consists not only in the morphological modification of bristle size but also in a striking prolongation of larval growth (Dunn and Mossige, 1937). The physiological mechanism underlying this developmental prolongation is unknown, but it seemed worthwhile to test whether egg cells derived from Minute females differed from egg cells derived from non-Minute females in their effect on the Dichaete reaction. (3) *pyd*. The use of this mutant was based on the supposition that its action toward increase of bristles may possibly result in a difference of egg cytoplasm from *pyd* as opposed to normal females.

The initial problem was to obtain seven stocks which were highly alike except for the mutants to be tested (*D*, *M-2*, *M-3*, *M-4*, *pyd*, and *vg*). The method was to replace whole chromosome "marked" with dominant mutant genes by homologous chromosomes of the isogenic wild type stock "Canton-S." A laboratory stock with dominant marked genes in the second and third autosome, of the genotype *+/+; Cy/Pm; Sb-C/H\** was crossed with each stock of *D*, *M-2*, *M-3*, *M-4*, *pyd*, and *vg* and their progeny outbred for three generations with Canton-S stock. In case of crosses involving *D* or the Minutes, when all marked chromosomes, as well as the X and Y, had been replaced by homologous Canton-S chromosomes, the mutant female of the resultant stock was chosen as one parent of each subsequent cross with Canton-S males. This was done in order to produce as much homogeneity as possible in the mutant chromosomes by allowing crossing-over to occur. The crosses described above were maintained for fourteen generations; subsequent flies were maintained by mass matings with all female parents of such mass matings selected from the same bottle. The Canton-S flies referred to were reproduced in each generation by pair matings from a single bottle.

A slightly different breeding method was used with *vg* and with *pyd*, because the former is a recessive and the latter a very weak dominant; it was necessary to inbreed flies from the fourth generation in order to obtain the *vg* or *pyd* phenotype (fully expressed in homozygous condition only). Thereafter, the *vg* and *pyd* flies were alternately outbred to Canton-S and inbred until the fourteenth generation was reached and the *vg/vg* and *pyd/pyd* flies were used in the experimental crosses.

The experimental flies were obtained from reciprocal crosses between the approximately isogenic stocks of Dichaete and of *vg*, *pyd*, *M-2*, *M-3*, and *M-4*. Reciprocal crosses were also made between Dichaete and non-Dichaete siblings. Altogether, twenty crosses involving 456 cultures were made.

All crosses were maintained in a seven-shelved incubator ventilated by a forced draft fan. The temperature was maintained between 24.5 and 25.0 degrees Centi-

\* Symbols used here refer to mutant genes as follows: *C* = crossing-over suppressor, *Cy* = curly wings, *H* = lack of certain hairs, *Pm* = plum eye color, *Sb* = stubble bristles. For further details see Bridges and Brehme (1944).

grade throughout the whole experiment. Crosses were indiscriminately mixed on the shelves, thus insuring that no one group of bottles occupied a "favored" position.

Two culture methods were used. (1) In the "bottle" method, virgin females were collected over a two-day interval and each was put with a male in a  $20 \times 90$  mm. shell vial containing a piece of agar-molasses substrate (unyeasted) to supply moisture. At the end of the two days, the pairs of flies were transferred to ordinary half-pint milk bottles with the usual cornmeal-molasses food (unyeasted). After an additional three days the parents were shaken out and the bottles returned to the incubator. (2) The "egg-count" method differs in a few respects from the "bottle" method. Virgin females were collected as before and pair-mated in  $20 \times 90$  mm. shell vials for two days. The pairs were then removed and put into half-pint milk bottles containing egg-laying dishes. These were rectangular metal dishes,  $60 \times 30$  mm. long and wide, and 10 mm. deep, filled with food. The same substrate was used as above. Egg counts were then made. When 120 eggs had been deposited on the surface of the substrate, 12-36 hours later, it was transferred to a  $4\frac{1}{2}$ -inch finger bowl with yeasted food medium. The bowl was then covered with cotton gauze and another finger bowl and placed in the incubator. When all larvae had pupated they were transferred to bottles and remained there until eclosion.

#### EFFECT OF CULTURE METHOD ON POPULATION DENSITY

Considerable variability in size of population per bottle and in average population per cross was found with either culture method. The area of substrate per larva (as judged by flies surviving to eclosion) also differed greatly. Table I contains relevant data.

TABLE I  
*Effect of culture method on population density*

	Egg count method. Area of agar surface 9710 mm. <sup>2</sup>		Bottle method. Area of agar surface 2633 mm. <sup>2</sup>	
	Total population surviving	Area per surviving larva	Total population surviving	Area per surviving larva
Least densely populated bottle	33	294	75	35
Least densely populated cross (average)	69	141	100	26
Average of all crosses	82	118	115	23
Most densely populated cross (average)	95	102	130	20
Most densely populated culture	123	79	189	14

The two methods offered very different conditions of existence to the larvae. If these conditions had any significant effect upon bristle number, identical crosses cultured by the two methods would not be expected to yield comparable results.

## ANALYTICAL METHODS

The Dichaete offspring of the experimental crosses were classified by sex and number of dorsocentral bristles. No distinction was drawn between  $M$  and  $M^+$  flies. As an index, the mean number of posterior dorsocentral bristles per hemithorax was computed separately for each sex in each bottle. In computing the index, one-half the number of one-bristle flies were added to the total number of two-, three-, and four-bristle flies, and this resulting sum divided by the number of flies of that sex in that bottle. This method of calculating posterior dorsocentral bristle frequency is justified by the data of Plunkett (1926) and Walker (1941). According to these workers, the effect of Dichaete is to remove the anterior dorsocentral bristles first and the posterior dorsocentral bristles secondarily, if the effect is strong enough. This means that if two bristles remain, they are practically always the posterior dorsocentrals, and if one bristle is present it is with about equal frequency the right or left posterior dorsocentral. As an overall measure of bristle frequency (in one sex) in a particular cross, a mean bottle index ( $\bar{P}_{\text{cross}}$ ) was calculated in the same manner as for a single bottle. A summary of cross indices is shown in Table II.

TABLE II

*Bristle frequencies (as measured by presence of posterior dorsocentrals per hemithorax; wild-type = 1) for Dichaete progeny of the experimental crosses. "A" and "B" refer to reciprocal crosses, the former denotes the cross in which the female parent is Dichaete and the latter denotes the cross in which the male parent is Dichaete.*

Experimental cross number	Mutant used to test for "maternal" effect	Female progeny		Male progeny	
		A	B	A	B
		Mutant father	Mutant mother	Mutant father	Mutant mother
		Cross index		Cross index	
1A and 1B	vg (bottle method)	0.704	0.752	0.529	0.635
**2A and 2B	vg (egg-count method)	0.774	0.710	0.681	0.403
3A and 3B	M-4 (bottle method)	0.432	0.363	0.289	0.180
4A and 4B	M-3 (bottle method)	0.585	0.601	0.423	0.445
**5A and 5B	M-3 (egg-count method)	0.824	0.903	0.625	0.605
6A and 6B	M-2 (bottle method)	0.669	0.529	0.500	0.425
7A and 7B	pyd (bottle method)	0.697	0.596	0.660	0.445
8A and 8B, 9A and 9B, 10A and 10B	Dichaete* (egg-count method)	0.855 0.779 0.843	0.853 0.793 0.891	0.783 0.599 0.671	0.718 0.523 0.709

\* These experiments were carried out within the Dichaete stock itself; D/C females and males were crossed reciprocally with C/C males and females.

\*\* These crosses done by the "bottle" method and repeated later by the "egg-count" method.

Whether the indices (for the same sex) of the reciprocal crosses were or were not significantly different was determined in three steps:

(1) for each sex ( $\bar{P}_{\text{females}}$  and  $\bar{P}_{\text{males}}$ ), a joint mean of the two reciprocal crosses was calculated from the combined data of the two crosses in the same manner as for a single bottle.

(2) the bottles above and below the joint means, sexes considered separately, were entered in a four-fold table.

(3) this table was tested for homogeneity by the usual  $\chi^2$  method. The relevant data for all crosses are summarized in Table III which shows the probability

TABLE III

$\chi^2$ ,  $n$ , values for distribution of bottles whose indices fall above and below the joint mean ( $\bar{P}_{\text{females}}$  and  $\bar{P}_{\text{males}}$ ) for each reciprocal cross.

Crosses compared				Female progeny		Male progeny		Combined progeny	
No	♀	×	♂	( $n=1$ ) $\chi^2$	P	( $n=1$ ) $\chi^2$	P	( $n=2$ ) $\chi^2$	P
1.1 *1B	D/C vg/vg	×	vg/vg D/C	5.127	0.025	4.282	0.042	9.409	0.010
*2A 2B	D/C vg/vg	×	vg/vg D/C	5.432	0.020	24.966	0.003	30.398	<0.010
*3A 3B	D/C M-4/C	×	M-4/C D/C	2.785	0.100	2.115	0.160	4.900	0.089
4A *4B	D/C M-3/C	×	M-3/C D/C	2.131	0.154	1.054	0.306	3.185	0.204
†5A **5B	D/C M-3/C	×	M-3/C D/C	3.343	0.072	0.016	0.900	3.359	0.190
*6A 6B	D/C M-2/C	×	M-2/C D/C	9.185	0.007	6.417	0.011	15.602	<0.010
*7A 7B	D/C pyd/pyd	×	pyd/pyd D/C	13.616	<0.010	18.169	<0.010	31.885	<0.010
*8A 8B	D/C C/C	×	C/C D/C	0.744	0.407	3.702	0.056	4.446	0.115
†9A **9B	D/C C/C	×	C/C D/C	0.628	0.444	1.563	0.219	2.191	0.392
10A *10B	D/C C/C	×	C/C D/C	3.478	0.067	0.965	0.332	4.443	0.117

\* Cross giving higher index for progeny of both sexes.

\*\* Cross giving higher index for female progeny.

† Cross giving higher index for male progeny.

that such a distribution above and below the joint index could be a chance occurrence.

Of the mutants tested, three—*pyd*, *vg*, and *M-2*—have significantly different indices in reciprocal crosses; two, *M-3* and *M-4*, do not, nor does *Dichaete* itself.

One totally unexpected feature of these results is that the difference between reciprocal crosses involving *vg* was of opposite sign in the two trials, but significant in each case. In crosses  $1A(D/C\bar{q} \times vg/vg\delta)$  and  $1B(vg/vg\bar{q} \times D/C\delta)$  both male and female offspring had higher indices where the vestigial parent was the *mother*; in crosses  $2A(D/C\bar{q} \times vg/vg\delta)$  and  $2B(vg/vg\bar{q} \times D/C\delta)$  each sex showed a larger index where the vestigial parent was the *father*. Among many possible explanations, three seem most worthy of consideration. (1) Inefficient statistical methods may have been used. It may be recalled here that the general purpose of statistical tests of the reality of observed differences is to predict whether similar differences would be expected in repetitions under comparable conditions. Predictions are based here on the variation of bottle indices *within* an experiment. In the present case the statistical test of either *one* experiment of a pair leads to the expectation that the other experiment should yield a difference of the same sign, if it has been performed similarly. The other, however, gives opposite results. (2) There may have been a difference in procedure between the two experiments of a sort which, in general, reverses the direction of difference between reciprocal crosses involving *vg* and *Dichaete*. In fact crosses  $1A(D/C\bar{q} \times vg/vg\delta)$  and  $1B(vg/vg\bar{q} \times D/C\delta)$  were raised by the bottle method and had average population densities of 4.7 and 4.4 larvae per  $\text{cm}^2$  (as judged by the number surviving to eclosion); crosses  $2A(D/C\bar{q} \times vg/vg\delta)$  and  $2B(vg/vg\bar{q} \times D/C\delta)$ , raised by the egg-count method, had densities of 0.85 and 0.91 larvae per  $\text{cm}^2$ . (3) There may have been a difference in material between the two experiments. In fact the vestigial parents used in the two trials were not of undoubtedly identical genotype. In maintaining the *vg* and Canton stocks, a contamination was noticed in both of them between the first and last *vg* experiments. The Canton stock was re-obtained from a laboratory stock which had come originally from the same stock as the first Canton stock but had been inbred by mass matings instead of pair matings. A stock of vestigial was obtained from the same source as before and was made approximately isogenic by the same procedure as before. On the whole, then, explanation (3), difference in stock, seems relatively improbable.

Explanation (1), inefficient statistical method, also may reasonably be excluded: the method is standard and its prediction about repetitions is upheld in the one cross which was repeated without change in culture method. Three sets of reciprocal crosses were made between *Dichaete* and non-*Dichaete* siblings. In each set of reciprocal crosses the indices for both sexes were found not to be significantly different. The prediction from the statistical tests is also fulfilled in one case where the culture method was changed, that is, crosses  $4A(D/C\bar{q} \times M-3/C\delta)$  and  $4B(M-3/C\bar{q} \times D/C\delta)$  were done by the bottle method and their indices (for both sexes) were found not to be significantly different. Later, this same pair of reciprocal crosses was repeated (cross  $5A: D/C\bar{q} \times M-3/C\delta$  and cross  $5B: M-3/C\bar{q} \times D/C\delta$ ) and the progeny raised by the egg-count method. Here, as before when the bottle method was used, neither sex showed indices significantly different from those of their reciprocal crosses.

Thus explanation (2), effect of culture method, seems to be the only one of the three leading possibilities which cannot be reasonably excluded. That it



should be *expected* to bring about a reversal of the difference between reciprocal crosses is not obvious from the literature on bristle phenotypes. That it may actually have done so can scarcely be decided without some study of the relation between bristle index and the factors which change with varying culture conditions. It is with this problem that the following section is concerned.

#### FACTORS AFFECTING THE BRISTLE INDEX

Three factors affecting the bristle index will be considered.

(1) *Differences within crosses.* Whatever the factors may be which affect the bristle frequency in the present material, they do not operate identically even through a group of bottles prepared at the same time, containing progeny from parental pairs of the same genotype, and all incubated within 0.5 degrees Centigrade of the same temperature. This fact was established by making  $\chi^2$  tests for homogeneity for the female indices within each of the twenty experimental crosses. The results of these tests are listed in Table IV. Altogether, in twelve

TABLE IV  
 $\chi^2$  treatment of the comparison of bottle and group indices of the female progeny within crosses 1A-10B

Cross				$\chi^2$	n	P
No.	♀	×	♂			
1A	D/C	×	vg/vg	94.179	34	<0.010
1B	vg/vg	×	D/C	64.463	16	<0.010
2A	D/C	×	vg/vg	55.793	29	<0.010
2B	vg/vg	×	D/C	105.025	35	<0.010
3A	D/C	×	M-4/C	47.372	30	0.024
3B	M-4/C	×	D/C	12.815	17	0.747
4A	D/C	×	M-3/C	104.200	37	<0.010
4B	M-3/C	×	D/C	108.071	40	<0.010
5A	D/C	×	M-3/C	18.895	18	0.407
5B	M-3/C	×	D/C	9.082	14	0.823
6A	D/C	×	M-2/C	132.111	29	<0.010
6B	M-2/C	×	D/C	143.243	45	<0.010
7A	D/C	×	pyd/pyd	72.103	31	<0.010
7B	pyd/pyd	×	D/C	93.845	45	<0.010
8A	D/C	×	C/C	25.115	19	0.163
8B	C/C	×	D/C	18.893	17	0.339
9A	D/C	×	C/C	42.234	39	0.674
9B	C/C	×	D/C	71.068	36	0.013
10A	D/C	×	C/C	26.987	39	0.162
10B	C/C	×	D/C	72.094	41	<0.010

of the twenty crosses, significant differences among bottle indices for females were found between duplicate bottles of the same cross.

(2) *Differences related to eclosion order.* In addition to the unexplained differences among bottles treated as similarly as possible, consistent differences in bristle index are also found within individual bottles. In each of the crosses raised by the bottle method, the first flies to eclose showed a higher mean bristle

index than the flies included in the second count, two days later. And these in turn had a higher mean index than flies included in the third count which was four days after the first. That is, in general, the bristle index decreased with eclosion order.

The underlying factor or factors which relate the bristle number of an individual fly to its eclosion order are unknown. They may include, among others, amount of moisture of food, presence of metabolic wastes of larvae, and amount of yeast available during all or part of the life of a larva. The yeast growth of a particular culture presumably varies partly with the number of larvae which have previously been feeding upon it, and perhaps partly with elapsed time, independently of larval population.

(3) *Difference related to area per eclosed larva.* It was found in all but one out of sixteen cases that bristle index was positively correlated with substrate area per eclosed larva. The positive correlation coefficients were found to vary from 0.479 in the male progeny of cross  $2A(D/C♀ \times vg/vg♂)$  to 0.049 in the male progeny of cross  $2B(vg/vg♀ \times D/C♂)$  (Table V). The excessive variability

TABLE V  
Correlation coefficients between bristle index and substrate area

Cultured by	Cross	Female progeny	Male progeny
		<i>r</i>	<i>r</i>
Bottle method	1A ( $D/C \times vg/vg$ )	0.303	0.160
Bottle method	1B ( $vg/vg \times D/C$ )	0.471*	0.078*
Egg-count method	2A ( $D/C \times vg/vg$ )	0.099*	0.479*
Egg-count method	2B ( $vg/vg \times D/C$ )	0.085	0.049
Bottle method	4A ( $D/C \times M-3/C$ )	0.092	0.084
Bottle method	4B ( $M-3/C \times D/C$ )	0.309	0.321
Egg-count method	5A ( $D/C \times M-3/C$ )	0.328	0.257
Egg-count method	5B ( $M-3/C \times D/C$ )	-0.037	0.228

\* Signifies cross with significantly different mean bristle index in reciprocal crosses.

of indices within crosses, and the influence on size of index of eclosion order, makes difficult an evaluation of the correlation between index and substrate area. A clarification should be based on data containing counts of daily hatches and not only, as in the data used, on complete hatches.

#### DISCUSSION

The original purpose of this investigation was to test for "maternal effect," *i.e.*, phenotypic effects expressed exclusively through or by the maternal parent, in the offspring of reciprocally crossed isogenic stocks. No significant differences were found between reciprocal crosses involving *M-3*, *M-4*, nor within *Dichaete* itself, but significant differences between reciprocal crosses involving *M-2*, *pyd*, and *vg* were found (as judged by mean posterior dorsocentral bristle index). However, two groups of data showed large and unexplained variation. These were the significant differences in mean bristle index of cultures *within* crosses, and the reversal in sign of the significant differences between crosses  $1A(D/C♀ \times vg/vg♂)$  and  $1B(vg/vg♀ \times D/C♂)$ , and crosses  $2A(D/C♀ \times vg/vg♂)$  and

$2B(vg/vg \text{♀} \times D/C\text{♂})$ . In regard to the former, these significant differences arose among progeny of parental pairs of identical age and genotype. They arose in spite of efforts to produce, as nearly as possible, identical treatment of all flies. It must be concluded that these efforts were not sufficiently successful in making the environments of different sets of progeny homogeneous. Such factors as the condition of the substrate, the availability of yeast, and possibly the presence of bacteria and fungi in the fermenting medium were probably of influence on the bristle indices. With regard to the reversal in difference between bristle indices in the two sets of *vg*-Dichaete crosses, the unexplained variability just discussed may be the only cause of the opposing results. In addition, it should be pointed out that different culture methods were employed in the *vg*/Dichaete experiments. During larval life the closure of the experimental dishes in the "egg-count" method by means of cheesecloth and fingerbowls is considerably less tight than in the bottle method. It allows for more loss of substrate moisture and permits perhaps greater inoculation with bacteria and fungi. Furthermore, the greater area of substrate per larva in the "egg-count," as compared to the bottle method, presents a difference in environment, in degree if not in kind, to the developing larvae. The availability of yeast and the abundance of competitive organisms may be altered, as part of this environment, and in turn may influence the production of posterior dorsocentral bristles. Whether the effects of these two different environments is of such nature as to cause a significant reversal of mean bristle index between these two sets of reciprocal crosses is not conclusively shown in these data. The fact, however, that they may influence mean bristle index in experimental flies should not be ignored.

In the interest of more exact and uniform conditions, certain refinements of technique in future experiments may be mentioned here. It is well known that too few as well as too many larvae do not produce healthy culture conditions. Therefore, the optimal area per substrate per larva should be determined. In view of the fact that great variations in egg hatching are sometimes encountered, it is suggested that, where possible, counted larvae are chosen for experimental material rather than eggs. The substrate itself varies somewhat from culture to culture in the amount of moisture present and acidity (Bridges and Darby, 1933). Buffered substrate and controlled moisture conditions might aid in establishing more uniform culture conditions.

Differences between reciprocal crosses somewhat similar to those encountered in this work have also been described for *vg* by Hersh and Ward (1932), and Child (1939). No data were presented dealing with differences in wing size related to eclosion order, or to amount of substrate per larva, nor have tests of homogeneity been reported. The present work points to the necessity for extremely well controlled culture conditions and a searching statistical analysis, before conclusions concerning "maternal effect" can be accepted. In view of the residual, uncontrolled variability, the data obtained in this work are inconclusive as to the presence or absence of a "maternal effect."

#### SUMMARY

1. To test for the existence of maternal effects in *Drosophila melanogaster*, approximately isogenic stocks of Dichaete, *pyd*, *vg*, *M-3*, *M-4* were prepared.

Reciprocal crosses were made between each stock and Dichaete, and between Dichaete and non-Dichaete siblings. The results, as indicated by posterior dorso-central "bristle indices" (*i.e.*, average number of dorsocentral bristles per hemithorax), of the Dichaete progeny show that no significant differences arose within six pairs of reciprocal crosses. The reciprocal crosses of four other pairs of experiments yielded significant differences in bristle indices. Two of these pairs were alike in type of crosses, but different in type of culture method. The sign of the differences in bristle indices was reversed in these two sets of experiments, the progeny with the higher indices coming from Dichaete mothers in one case, and from Dichaete fathers in the other.

2. Tests of homogeneity show that within crosses, all bottles of which have had identical treatment, differences in mean posterior dorsocentral bristle frequency occur which are greater than those to be expected on the basis of simple sampling errors.

3. The size of the bristle index of an individual fly depends partly upon its eclosion order, with those flies that eclose earliest having the highest bristle index.

4. The size of the bristle index depends partly on area of substrate available to the individual, although this dependence may be weak in many cases.

5. The lack of homogeneity in indices within crosses, and the reversal in direction of "maternal effect" in the pairs of crosses grown under two different culture conditions, suggest that external conditions during the development of the flies must be made more constant than heretofore, before conclusions as to the presence or absence of a "maternal effect" can be drawn.

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## BINARY ANTERIOR OCELLI IN ANTS

NEAL A. WEBER

In a paper by this name in the *Biological Bulletin* William Morton Wheeler (1936) published an account of binary anterior ocelli which was believed to be the first record of this condition in adult insects. He was led into a search of the literature by finding a doubling of the anterior ocellus in 15 out of more than 4,000 of a colony of anomalous ants (*Cephalotes atratus quadridens* DeGeer), which he called gynandromorphs. This colony was one which I collected in Trinidad, British West Indies. The discovery led him to examine other ants, an examination which revealed a comparable condition in a soldier of the Indomalayan ant, *Pheidologeton diversus luotina* Santschi, and in more than 60 per cent of a series of 300 soldiers from 25 colonies of the neotropical leaf-cutting ant, *Atta cephalotes* L.

The anterior ocellus of the insect eye has long been known to have a double innervation, while the lateral ocelli have a single innervation. In the ontogeny of insects there are also two primordia for the anterior ocellus itself, one for each of the lateral ocelli.

It is the purpose of this note to give credit to a much earlier discoverer of the binary anterior ocellar condition in ants and to add several additional records.

H. W. Bates in "*The Naturalist on the River Amazons*," of which the first edition was published in 1863, while writing of the large leaf-cutting ants or Saüba at Pará, Brazil, says:<sup>1</sup>

"The third order of workers is the most curious of all. If the top of a small, fresh hillock, one in which the thatching process is going on, be taken off, a broad cylindrical shaft is disclosed. . . . If this be probed with a stick . . . a small number of colossal fellows (Fig. 3) will slowly begin to make their way up the smooth sides of the mine. Their heads are of the same size as those of the class Fig. 2; but the front is clothed with hairs, instead of being polished, and they have in the middle of the forehead a twin ocellus, or simple eye, of quite different structure from the ordinary compound eyes, on the sides of the head. This frontal eye is totally wanting in the other workers, and is not known in any other kind of ant."

A new record which is here figured (Fig. 1) is that of another fungus-grower, *Acromyrmex* (A.) *coronatus* Fab. In this genus, however, there is no soldier caste, although the workers are otherwise as polymorphic, and the large workers do not normally have ocelli. Three large workers from Bolivia (Rosario: L. Rocagua, W. M. Mann, collector) show the condition as represented. The frontal area,

<sup>1</sup> His description and figures (Everyman's Libr. ed., 1930, pp. 10-16) of the large, shiny-headed soldier with distinct ocelli fits *Atta cephalotes* L. and this is the species he names (as *Oecodoma cephalotes*). He, however, states that the male is not much more than half the size of the female and this is characteristic of *Atta sexdens* L., the common Brazilian species. It is possible that most of his extensive account, which is not quoted, refers to *sexdens* and the condition of double ocelli refers to *cephalotes*. When he returned to England he may have examined the latter in collections and then discovered the ocelli, or he may have seen the condition in life along the upper Amazon where *cephalotes* and *laevigata* occur.

above the convex posterior margin of the clypeus and between the bases of the antennal scapes, is shown. The ants are normally rugose here and this condition is indicated by short irregular lines. The dotted lines constituting the margin of each figure represent the frontal carinae which are expanded anteriorly as frontal lobes covering the insertions of the antennae. In the middle of each frontal area posteriorly lie the paired ocelli. Each pair is in the position of the normal single anterior ocellus of the functional female caste of the species. In the female of *A. coronatus globoculis* Forel, for example, the lateral ocelli lie immediately outside the frontal carinae in the position indicated in the figures by a pair of short, converging, dotted lines forming the most posterior portion of the drawings.

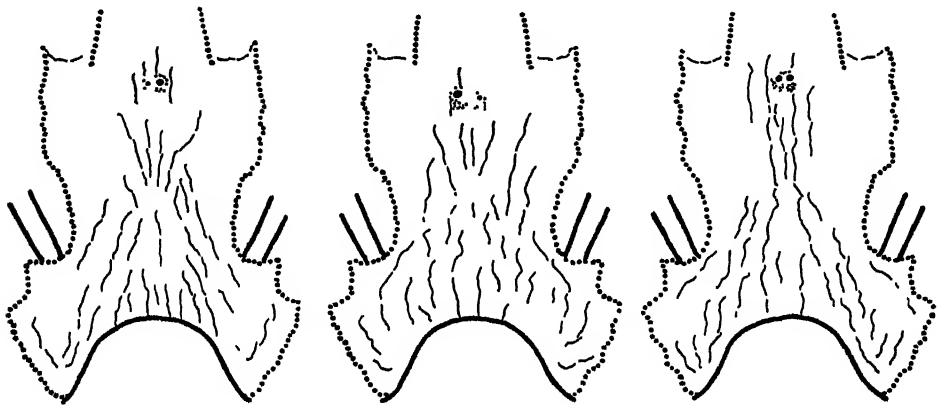


FIGURE 1

Other species of fungus-growers in my collection show binary anterior ocelli or other variations from the usual absence of ocelli.

Soldiers of *Atta cephalotes* from Peru show the condition described by Dr. Wheeler. The three mounted maxima soldiers of the type colony of *A. cephalotes isthmicola* Weber from Panama have large binary anterior ocelli and vestigial or no lateral ocelli. Three smaller soldiers of the same colony have each a minute anterior ocellus and no lateral ocelli.

Out of three soldiers of *Atta sexdens rubropilosa* Forel (det. Gonçalves) from Rio de Janeiro, only the largest shows ocelli and these are a minute lateral pair. Three soldiers of *A. sexdens robusta* Borgmeier from Rio de Janeiro (Gonçalves) show minute anterior and much larger lateral ocelli.

Large soldiers of *Atta laevigata* (F. Smith) from Venezuela, Brazil and Bolivia have a variable anterior ocellus which in some is smaller, in others larger, than the lateral ocelli. Several of the anterior are binary.

It may be pointed out that all of these records, now comprising four genera, are from the subfamily Myrmicinae which occupies a position about midway in the phylogeny of ants.

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# AN ELECTRON MICROSCOPE STUDY OF PROTOZOAN FLAGELLA

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## INTRODUCTION

While the magnifications obtained by the electron microscope have added greatly to the knowledge of the morphology of bacteria, the protozoa have been found to be too thick for easy penetration (unpublished work). However, it was believed that certain protozoan structures could be studied to advantage, and the following paper summarizes some results of electron microscope studies of flagella.

Flagella have been studied extensively with the light microscope. Vlk (1939) demonstrated several types among which was the ciliary type consisting of a shaft on which were located numerous small cilia, or very fine fibrils. Other biological fibers have been studied and are being investigated by means of the electron microscope. Jakus (1945) studied the morphology of trichocysts of *paramecia* elucidating the "ultra-structure" of the shaft. The shaft material is made of fibers which show long spacings typical of contractile fibers. These spacings, which probably represent the fundamental molecular arrangement in the fiber, differ slightly in length from the spacings in myosin and collagen. The connective tissue fiber, collagen, has been studied by Schmitt, Hall, and Jakus (1942) using the electron microscope. They were able to measure directly the long spacings which had previously been studied by Clark, Parker, Schaad, and Warren using X-ray diffraction techniques (1935), and had been remeasured by Bear (1942, 1944). Hall, Jakus, and Schmitt (1945) demonstrated several distinct long spacings in muscle fibers.

Fibers have been observed by electron micrograph in the tails of sperm by Baylor, Nalbandov, and Clark (1943) using bull sperm, and by Harvey and Anderson (1943) using the sperm of the sea urchin. The axial filament of the sperm tail in unfixed preparations separated into about ten to fifteen fibrils either in the region of the naked filament or in the region of a break in the protoplasmic sheath. The flagella of bacteria have been studied by Mudd, Polvitsky, and Anderson (1942) using the electron microscope.

By all odds the outstanding investigation on flagella, both in structure and function, is that of Harley P. Brown (1945). This Ph.D. thesis gives an exhaustive historical background as well as an account of the author's extensive experiments ranging from electron micrographs to underwater swimming. This paper unfortunately did not come to the attention of the present authors until a considerable amount of our work had been completed. Actually, there is very little to be added to the work of Brown. But it seems of value to record our independent observations, most of which are on quite different organisms. Our electron micrographs in part confirm the data of Brown, but those included in this paper primarily present new information on flagella not disclosed in his electron micrographs.

Brown studied the following organisms: *Astasia kelebsii* Lemmermann, *Euglena gracilis* Klebs, *Ochromonas variabilis* Meyer, and *Chilomonas paramecium* Ehrenberg. The micrographs show that the flagella are of approximately uniform diameter throughout their entire length; that each flagellum consists of a denser axial core (axoneme) and a less dense sheath surrounding the core; that in the flagella of *Euglena* and *Astasia* the axial core appears to consist of two fibers of equal size; that the sheath appears to contain or consist of a coiled fiber in a helix encircling the axial core; that flagella of *Euglena* and *Astasia* bear along one side a single row of delicate filaments, designated flimmer, while the long flagellum of *Ochromonas* bears similar flimmer along both sides, and that of *Chilomonas* bears none. The general conclusion of Brown is that the flagellum beats in spiral undulations, confirming the theory of Lowndes (1945).

#### MATERIALS AND METHODS

The R.C.A. electron microscope, type B, was used in the following experiments. The microscope and its application to biology has been described in detail by Marton (1941), and by Zworykin, Marton, Ramberg, Hillier and Vance (1945). The specimen was placed directly on a collodion membrane supported by a standard wire mesh which served as the "slide." In the experiments which follow it was often desirable to examine the specimens at low magnifications so as to correlate the results with those of the light microscope. It was also desirable to view a wide field. The wide field, low magnification and greater depth of focus were obtained by placing the "slide" in the top of the specimen holder. The optical principles involved have been described by Burton, Barnes, and Rochow (1942). The technique of calibration described in that paper was employed to obtain the magnification figures used in this work.

Mixed cultures of protozoa were obtained from water from the Boneyard Creek in Urbana, Illinois. They were subcultured in chlorine-free water to which a grain of unpolished rice was added. Some pure cultures were attempted by subculturing from single organisms picked from dilute mixed cultures by fine pipette, but results of this procedure were unsatisfactory both as to photographs obtained and purity of culture. Saline suspensions of the gut contents of the cockroach *Periplaneta americana* were prepared in an attempt to photograph the flagella of the flagellates *Lophomonas blattarum* and *L. striata*. In a similar manner, suspensions of the gut contents of the termite *Reticulitermes flavipes* were prepared in order to observe the flagella of this rich flagellate fauna.

Specimens were prepared in the following ways:

1. A drop of culture was placed directly on the collodion membrane and allowed to dry in air. Some of these were washed in distilled water to remove excess debris, organisms, or salt.
2. A drop of the culture was placed directly on the collodion membrane, allowed to stand five to ten minutes in air, and then before complete drying had occurred, the screen was touched to a meniscus of distilled water. The surface tension of the meniscus drew the liquid from the screen leaving the biological material attached to the membrane.
3. Organisms were fixed in formalin directly on the screens, which were then washed in the above manner to remove the excess formalin.



4. Mixed cultures were fixed and stained with osmium tetroxide by adding 2 per cent  $\text{OsO}_4$  in aqueous solution to the drop containing the organisms, allowing the organisms to sink, and then pipetting them to the screen.

### RESULTS

Most of the protozoa were too large and thick for penetration by the electron beam. Specimens of *Euglena* sp. allowed no penetration, either in the region of the cell body or through the flagellum itself. Preparations from the gut of both cockroach and termite contained sufficient extraneous matter to obscure the structural details of the locomotor organelles of the flagellates. However, flagellates from the fresh-water cultures, some of which were tentatively assigned to the genus *Monas*, did allow enough penetration after drying to permit the cell outline and granules within the cell to be observed. Since this favorable material possessed several different and distinct types of flagellar forms, it was used for most of the studies herein described.

The general cell form of the organisms studied can be observed from the electron micrographs. Figure 1 shows a round cell body of *Monas* sp. which has undoubtedly flattened and shrunk during drying. The cell membrane is clearly seen, as is the protoplasmic mass which has become distorted during drying. Numerous granules can be seen within the cell, but their identification can only be surmised. The unidentified flagellate in Figure 2 shows an invagination at the apex behind which is a clear area, suggesting a cytostome and reservoir, or perhaps a contractile vacuole.

The fixation techniques employed in this work were not of a nature to demonstrate delicate cellular or flagellar components. Denaturation of protein appears to render it more opaque to the electron beam as does certainly the addition of a heavy metal. Formalin fixation caused adherence of foreign material to the cell body. The effects of osmium tetroxide can be seen in Figures 4, 6, 7, and 10. Much debris is always adsorbed and the cell body is completely impenetrable. The freezing-drying technique (Wykoff, 1946) is suggested as being probably the best to preserve the form of these animals for study with the electron microscope.

The flagella seen in the accompanying photographs are of two types, the ciliary flagellum and the fibrous flagellum. Vlk (1938) named the ciliary flagellum and found it present in many species of flagellates. He called the type without cilia the whip-flagellum, which may be the same as the type observed in this work and designated as the fibrous flagellum owing to the nature of its structure. The ciliary type seen in Figures 1 through 7 appears to consist of a shaft or tube, or mass of densely packed elongate fibers surrounded by radially placed ciliary structures (or flimmer). The exact nature of the central shaft cannot be determined from the present photographs. At the distal end of the flagellum shown in Figure 1, and in the region of the bend in Figure 5 long heavy fibers may be seen. The shaft may be a tube of these, or may have them embedded in the tube wall as a contractile element. In Figures 2 and 3 the shaft appears solid. In Figure 7 the central structure, which has been fixed with osmic acid, may possibly be interpreted as a hollow tube. It is probable that there are several different sub-types of the ciliary type of flagellum. In numerous cases one of the two flagella measured less than one micron in length. Flagella usually measured from 5 to 10 microns in length, and about 0.3 microns in width.

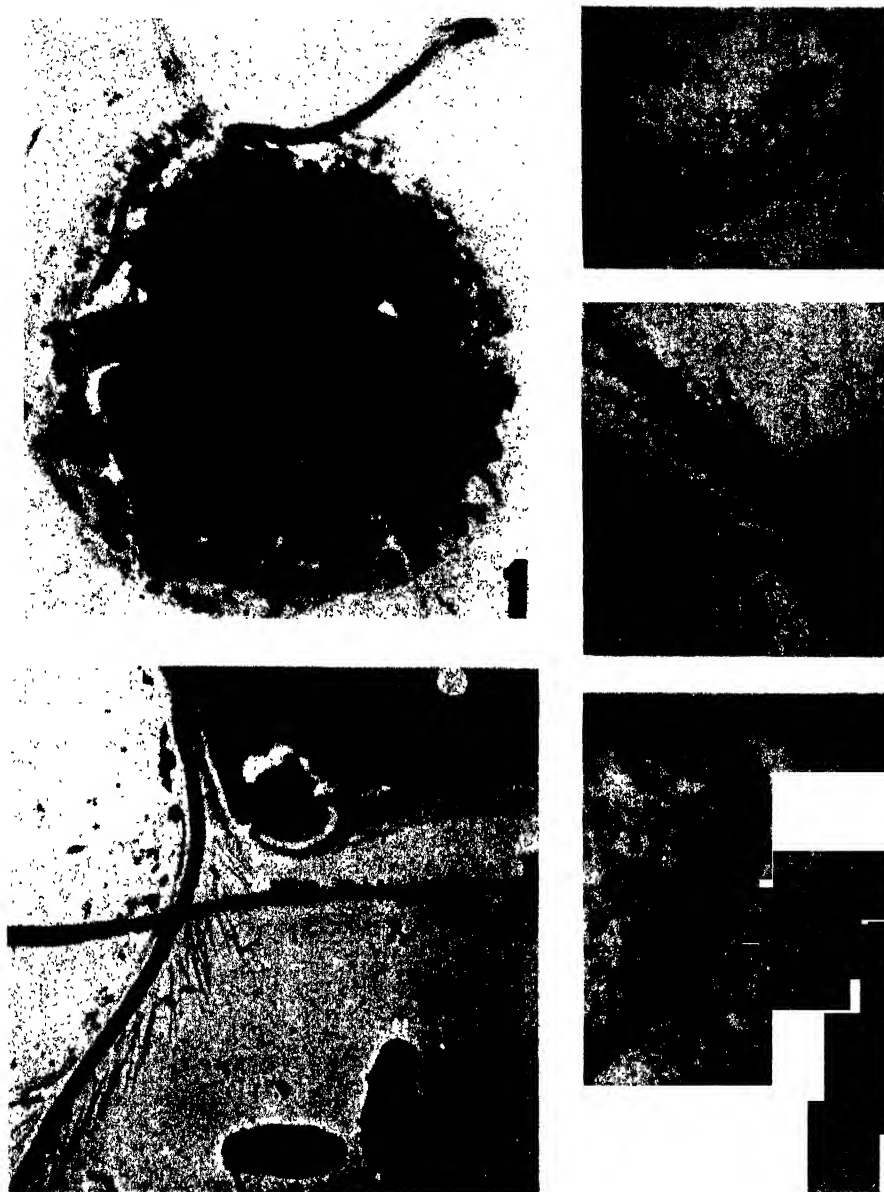


PLATE I

FIGURE 1. Electron micrograph of "*Monas* sp." showing cell body and attachment of two types of flagella (ca. 5,700  $\times$ ).

FIGURE 2. Organism (unidentified) showing single ciliary flagellum. Note shaft nodules and arrangement of cilia (ca. 4,700  $\times$ ).

FIGURE 3. Portion of ciliary flagellum showing nodules of shaft and cilia (ca. 6,600  $\times$ ).

FIGURE 4. Ciliary flagellum after osmic acid fixation (ca. 8,400  $\times$ ).

FIGURE 5. Ciliary flagellum with fibrous shaft (ca. 9,600  $\times$ ).

The point of attachment of the cilia on the flagella could not be determined by these photographs. Figures 2 and 3 show small nodules limited to one side of the shaft from which groups of cilia appear to take their origin. These are most dense in the proximal region. Brown ascribed this appearance to the helical fibrous sheath closely appressed to the axial core. Figures 1 and 5 show no such nodules although the cilia are clearly seen. In Figure 4 the form of the shaft is obscured by clumps of osmic acid which have also deposited between the individual cilia.

The fibrous flagella are best seen in Figures 8 and 9. They appear to be long fibers twisted spirally as the fibers of a rope. The rope-like quality is especially clear in Figure 9 where the tip of the flagellum has frayed. This structure was not observed in the microscope of Brown. Figure 8 shows the base of a flagellum which has broken away from the rest of the cell. These were not uncommon. The fibers appear to extend into the circular portion which is probably a part of the cell body. It would be interesting to observe isolated portions of the cell membrane for long fibers.

Long spacings were sought in the fibers. Enlargement of the fiber showed none. However, only very excellent photographs at high magnifications and at perfect focus can be used for these determinations. In osmic acid fixed rat tail collagen we were able to observe the long spacings reported by Schmitt, Hall and Jakus (1942). Hall, Jakus and Schmitt (1945) were able to demonstrate spacings in myosin only after treatment with either osmic acid or phosphotungstic acid. Figure 10 is a high magnification of the flagellar fibers treated with osmic acid. The granulation seen in the fibers is very regular, and paired rows of granules appear to lie together. This is the only indication of a periodicity which has been found in this work. It may be an artifact caused by precipitated osmic acid enmeshed in the fibrils. Very careful additional work should be carried out in this direction.

#### DISCUSSION

The agreement of the gross morphology of protozoans as revealed by electron micrographs with that seen by light microscope studies suggests that additional information may be gained by the electron microscope. The danger of artifacts introduced by the necessary treatment for such observations can be overcome by careful studies of morphological changes under different treatments and can be at least reduced to the level of that found in treatment preliminary to light microscope examination. The use of stains, in some cases specific, which are suitable to the optics of the electron system has been attempted, and will no doubt be developed to a satisfactory state. (Baylor, Appleman, Sears and Clark (1945) state that the electron "stain" must differentially alter the densities of various protoplasmic constituents.) The present day viewpoint of the necessity of knowledge of the specificity of a stain, and its action on well defined chemical constituents, ought to preserve the new field of electron microscopy from the array of sometimes meaningless colors now burdening light microscopy.

Needless to say, there is real need for careful identification of the specimens examined. Such was not possible at the time of this work. The necessity for pure cultures in investigations such as these is evident.



## PLATE II

- FIGURE 6. Two unidentified flagellates after osmic acid fixation (ca. 4,500  $\times$ )
- FIGURE 7. Osmic acid fixed ciliary flagellum, the shaft appears hollow (ca 17,000  $\times$ )
- FIGURE 8. Fibrous flagellum broken away from the cell body (ca. 7,500  $\times$ )
- FIGURE 9. Fibrous flagellum showing rope-like arrangement of fibers (ca. 7,500  $\times$ ).
- FIGURE 10. High magnification of frayed fibrous flagellum after osmic acid fixation (15,000  $\times$ ).

A mechanism of flagellar movement is suggested by the electron micrographs. The presence of fibrils in both types of flagella suggests that contraction and relaxation occurs as it is believed to occur in the fibers of muscle and connective tissue, that is, by molecular rearrangement. Contraction of individual fibers, particularly those arranged in the rope-like fashion seen in Figure 9, could account for the sweeping spiral movements observed in the living state. There is an indication that the fibrils of the fibrous type may extend into the cell body. Unilateral contraction of those in the cell body could certainly account for a variety of movements. The cilia of the ciliary type may function in either the original sweep or the recovery. They may also function in creating favorable currents in the water environment. In Figure 5 the cilia appear to be an extension of the protoplasmic membrane, and since no actual fibers can be observed within them, it may be that they serve to increase the sensory, absorptive or secretory surface of the cell.

The fibrous constitution of certain of the flagella indicates a broad distribution of fibers throughout the animal kingdom, from these simple organisms up through the complexities of the highly developed mammals. Are the fiber types the same throughout the whole kingdom? Is collagen, for example, present in all living animal forms, or does some other fiber take over its function in other species? Does the fiber form determine the function? Solution of these problems awaits further investigation and development of new and improved techniques.

#### SUMMARY

Certain flagellated protozoa have been photographed by the electron microscope. Two well defined types of flagella have been observed, a fibrous type in which the individual fibers occur in a twisted rope-like arrangement, and a ciliary type on which are arranged numerous small cilia. The shaft of the ciliary type in some cases appears to consist of fibrils. These observations verify in part and augment the masterly work of Harley P. Brown at The Ohio State University.

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# THE ROLE OF SNAPPING SHRIMP (CRANGON AND SYNALPHEUS) IN THE PRODUCTION OF UNDERWATER NOISE IN THE SEA<sup>1</sup>

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## INTRODUCTION

The sea has long been looked upon as a realm of silence. That this view is no longer tenable has been abundantly demonstrated as a result of extensive investigations with modern underwater sound detecting gear. These investigations, which during World War II became of grave importance because of submarine warfare and harbor defense, have shown that great stretches of numerous coastal areas are exceedingly noisy, periodically or perpetually, due to marine animals. Conversely, the investigations also indicate that vast areas of the deep ocean and of shallow water in higher latitudes are indeed notably silent.

The most widespread by far of all the biological underwater noises now known to exist in the sea is a crisp, high frequency, crackling noise caused by snapping shrimp. Examples of these animals are shown in Figures 1 and 2. It was not, however, until the second world war that the extent and persistence of this strange noise was realized and its cause determined. It has now become widely known as "shrimp noise" or "crackle."

The present report is intended to discuss, mainly, the cause and extent of crackle and to present the biological implications that explain its persistence and geographical distribution. Full details of the spectrum of the sound will be dealt with in another report (Everest, Young, and Johnson, in press).

## EARLY HISTORY OF SHRIMP NOISE

Before discussing the recent investigations that led to identification of crackle, it will be instructive to review briefly some of the early references that no doubt pertain to this noise phenomenon.

1. Mariners operating small vessels in tropical waters have sometimes reported hearing strange crackling noises within the holds of their craft. The most common

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<sup>1</sup> The work forming the basis of this report was done under contracts (NEMsr-30 and NObs-2074 respectively with the Office of Scientific Research and Development (Section 6.1 NDRC) and the Bureau of Ships, Navy Department, while the authors were members of the staff of the University of California Division of War Research at the U. S. Navy Radio and Sound Laboratory, San Diego, California. The following organizations cooperated in the collection of much of the data upon which this report is based: Navy Department, especially the Bureau of Ships; Naval Ordnance Laboratory; the U. S. Navy Radio and Sound Laboratory (now the U. S. Navy Electronics Laboratory); the Scripps Institution of Oceanography; the Coast Guard; and the Columbia University Division of War Research.

explanation given was that the noise was caused by shipworms (teredos) working in the ship's timbers, or that it was due to superficial fouling growths on the hull. It is now known that the crackle from nearby shrimp beds is audible through the hull and probably forms the basis for these stories.

2. The U. S. Coast and Geodetic Survey encountered troublesome noises while engaged in radio acoustic ranging off Oceanside, California in 1933-34. The sound was described as being similar to static crashes or to coal rolling down a metal chute. No explanation could be found for the noise, though efforts were made to correlate it with meteorological and hydrographic conditions, and with road traffic on a highway adjacent to the coast (Comdr. O. W. Swainson, 1943, personal correspondence). Subsequent surveys have demonstrated that shrimp crackle is conspicuous in the area where these observations were made, and is loud enough in some spots to be audible to the unaided ear above the surface during calm water. Dredgings, also, showed the animals to be present in the shale bottom, which is honey-combed by molluscan bores.

3. A U. S. submarine operating in Macassar Strait off Balikpapan early in 1942 circled an area from which a strange crackling noise was emanating. Lt. Comdr. W. D. Wilkins, in reporting this experience, suggested that "the Japs may have some newfangled gadget that they drop." Snapping shrimp are common in the Balikpapan region and the type of bottom and depth of water off Balikpapan form a favorable habitat for them. Other reports from submarine officers strongly suggest that noises often encountered in coastal waters in lower latitudes are attributable to shrimp.

4. Sounds were heard through the hull of a small vessel in Beaufort Harbor, N. C. and in calm water off Cape Hatteras (Hulbert, 1943). These sounds were described as similar to that produced by "dragging a blackberry vine." No doubt they were produced also by snapping shrimp for these animals are abundant in Beaufort Harbor and are now known to be very noisy there. They also occur in offshore waters at Cape Hatteras (Hay and Shore, 1916).

To summarize the above as well as other data, it is apparent from what is now known that the crackling noise has been attributed erroneously to various other phenomena. Some of these are (a) static crashes, (b) rolling pebbles, (c) surf, (d) volcanic or terrestrial disturbances, (e) shipworms working in a wooden hull, (f) superficial fouling on the hulls of ships, (g) noises due to expansion or contraction of the ship's structure, and (h) clapping of oyster or clam shells.

The sharp "snap" produced individually by these shrimp when captured has long been known, and has often been heard during low tide in some areas. The sound has hitherto, however, been considered only a biological curiosity and not a source of significant underwater background noise. Biological sounds similar to shrimp noise will be discussed later.

#### \* RECENT INVESTIGATIONS

Early in 1942 investigations of underwater ambient noise were begun by the University of California Division of War Research in the San Diego region. From the beginning of field tests a characteristic crackling noise of great magnitude was observed. It was first encountered at various locations off Point Loma and in the San Diego Yacht Harbor, and was found later in coastal waters off La Jolla, Ocean-



side, and other places on the southern California coast. The origin of the noise was a profound mystery until the end of 1942 when the cause was finally determined.

Identification of the noise was accomplished through laboratory tests of various animals and through field studies in many different habitats and over different populations. In the laboratory the animals were usually tested in large concrete tanks supplied with running sea water. Large public aquaria were also used.

For the listening tests and the exploratory studies, a simple crystal hydrophone and battery operated amplifier system sufficed. The equipment used for sound spectrum measurement in the field consisted of a non-directional hydrophone, a sound analyzer which was capable of selecting the various frequency bands (usually 50-cycles wide or a half-octave), and a graphic level recorder.

The sound emitted by an individual shrimp is a single sharp "snap" or "crack" produced only occasionally. It is the combined snapping of the members of a large population that results in a continuous loud underwater crackle over or near shrimp beds. In the audible band the crackling sound is comparable to the explosive noises produced by brisk burning of large quantities of dry twigs or frying of fat. Similar sounds can be heard by heterodyning ultrasonic bands in the 20-50 kc region down to the audible range. With increasing distance from the source, the sounds merge into a sizzle and finally a hiss without distinct cracks.

Sound spectrum analysis of crackle in the sea reveals that the shrimp noise has its strongest components in the higher frequencies. In contrast to shrimp noise, the noise produced by fish (Loye and Proudfoot, 1946, and Dobrin, 1947) is made up of low frequency sounds and is markedly seasonal and diurnal. In normal "water noise" resulting from waves and streaming of water, the maximum energy is also in the low frequencies. Because of this, shrimp noise is easily distinguished from both fish noise and water noise.

Figure 3 gives an idealized average of the sound spectra observed over shrimp beds in a variety of places. The ordinate for the sound measurements is sound pressure spectrum level.<sup>2</sup>

For comparison, the dashed curve shows the typical water noise observed in the deep sea, where there are no biological noises, for a state 1 sea.<sup>3</sup> This water

<sup>2</sup> Sound level is given as a number of decibels (db) above some reference value which in the present work is a root-mean-square sound pressure of 0.0002 dyne/cm<sup>2</sup>. This reference pressure is commonly incorporated in the calibration of sound-measuring instruments, but is frequently not explicitly stated. As a consequence, the decibel appears to be used with two meanings: a unit for an absolute measurement (with a reference value stated or only understood) and a unit for the *relation* of two sounds. In this paper when a sound level is stated in decibels it is in the first sense; the second sense is meant when a *change* or *difference* is given in decibels.

The decibel (db) is a logarithmic measure of power ratio (or ratio of pressure squared) such that a 100-fold increase in power (or 10-fold increase in pressure) corresponds to 20 db. A doubling of power is an increase of 3 db. A doubling of sound pressure is an increase of 6 db. The smallest change in noise readily perceptible to the ear is of the order of 1 or 2 db.

The noise being dealt with here is conceived to be comprised of a rather continuous distribution of sound vibration frequencies. The words "pressure spectrum level" refer to the effective sound pressure level for that sound energy contained in a band 1 cycle per second wide centered at the frequency indicated.

<sup>3</sup> The "sea" as used here refers to waves which are still growing because of the wind, and as such represent an additional motion superimposed upon the swell. A state 1 sea corresponds to waves from crest to trough less than 30 cm. high. See *Instructions to Marine Meteorological Observers*. U. S. Weather Bureau No. 1221, June 1941, p. 54.

noise increases at all frequencies with increasing roughness of the sea. It is apparent that at the lower frequencies the ambient noise over a shrimp bed is largely determined by the water noise found everywhere in the sea. However, above 2,000 cycles per second shrimp noise completely overrides the usual water noise. Above 10,000 cycles per second (10 kc) shrimp noise is some 30 db above the noise of a state 1 sea. The shrimp noise in the region of 10 to 20 kc exceeds the noise in a quiet sea by roughly the same amount as the audible noise in a factory exceeds the usual noise in a quiet residence without radio in operation. Because shrimp noise is greater than the water noise by such appreciable amounts, it is convenient to use an average spectrum level in the vicinity of 10 kc in studying noise suspected of being caused by shrimp. An average of the levels at 3, 5, and 10 kc has been used for this purpose, and also an average at 10, 15, and 20 kc; the latter has been arbitrarily selected for the present paper.

Analyses were made, also, of single shrimp snaps by taking oscillograms of the sound from isolated identified specimens. The shrimp were placed in a gauze net within a rubber bucket filled with sea water. The whole assembly was lowered into a fresh water lake, then the net was drawn from the bucket by means of a cord. Contact with fresh water stimulated the shrimp to snap and photographs were made of the snap impulses shown on the oscillograph screen.<sup>4</sup> The whole snap lasts about  $\frac{1}{2}$  to 1 millisecond. Further details are to be found in the acoustical report (Everest, Young, Johnson, in press). It is sufficient to note here mainly that the spectrum of a single snap, derived by a Fourier integral analysis, agrees qualitatively with the average spectrum over a shrimp bed.

It is not possible to reproduce in the laboratory the extent of crackle heard over shrimp beds in nature, or to simulate the hiss heard at a distance from the bed. Therefore, corroborative evidence must come from correlated field observation and spectrum measurements as discussed later.

The explanation of the local underwater sound mystery shed light on a number of qualitative reports of the type discussed in the previous section. Ultimately, it made possible the prediction of this type of ambient noise in other areas.

Sound surveys made since 1942 in widely separated areas along the East and West coasts of the United States from Key West to Chesapeake Bay and San Diego to Puget Sound, in the Bahamas, and in the Central and Southwest Pacific have confirmed the predicted, widespread distribution of shrimp crackle in localities that are ecologically suitable for snapping shrimp, and its absence in other situations. In the following section an explanation of the relationships is given.

#### THE BIOLOGY OF SNAPPING SHRIMP AND ITS RELATION TO SOUND LEVELS

The geographic locations of noisy crackling areas in the sea and the stability of the sound in these areas are so closely bound to the snapping habit and to the living requirements of these animals that it is necessary to consider their biology in some detail in order to elucidate these problems.

##### *Biological relationships*

Technically, the snapping shrimp are related to the commercial shrimp, which they resemble in general appearance. In life habits and details of structure, how-

<sup>4</sup> The assistance of T. F. Johnston and T. McMillian in this phase of the work is gratefully acknowledged.



FIGURE 1. *Synalpheus lockingtoni* natural size. Photographed from live specimens. At upper left: specimen with claw open to snap



FIGURE 2. *Crangon californiensis* 2♂.

ever, they are very unlike the common shrimp. The snapping shrimp family Crangonidae (Alpheidae, see Rathbun, 1904) comprises about 27 genera and numerous species. Of these only the species of two genera, viz., *Crangon* (also called *Alpheus* by many authors) and *Synalpheus*, are capable of vigorous snapping. In the literature there are recorded about 215 species of *Crangon* and 150 species of *Synalpheus*. Two species of these genera are shown in Figures 1 and 2.

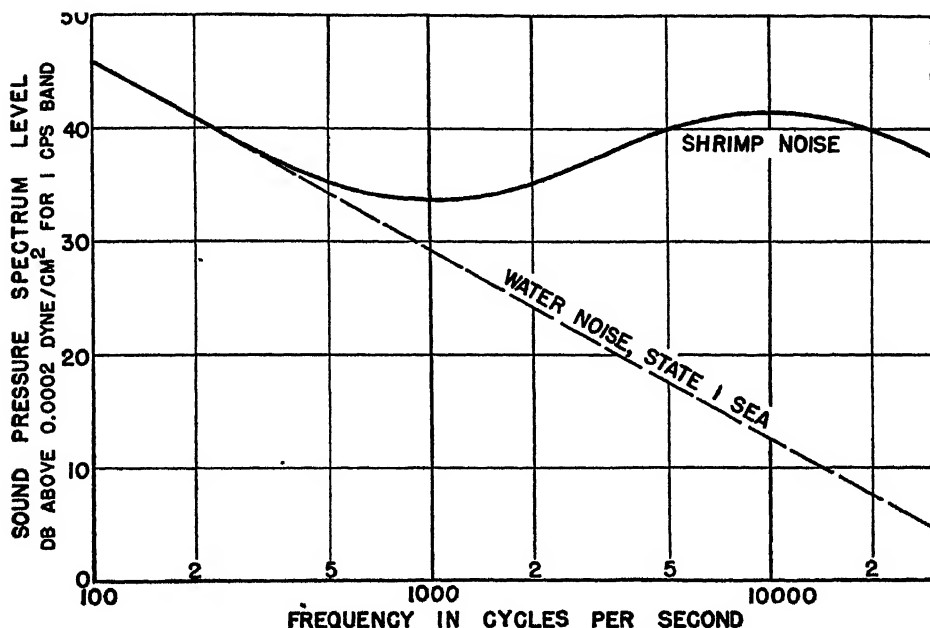


FIGURE 3. Average ambient noise spectrum over shrimp bed.

### *The snapping habit*

Studies of most species of these two genera have been based on preserved specimens; consequently, the snapping has not been observed directly in many species. The claw structure, however, indicates that all the species do snap to some extent. The species range in size from about 2 cm. to a giant species, *C. strenuus*, attaining a length of 8 cm., but it is clear that size is not necessarily correlated with the noise produced. The few direct sound measurements available of isolated specimens indicate, for example, that *Synalpheus lockingtoni* may produce a louder snap than its larger relative *Crangon dentipes*. The average peak pressure level of *Crangon* for a distance of a meter was 115 db above 0.0002 dyne/cm.<sup>2</sup>; whereas it was about 124 db for *Synalpheus*. While the difference may be real, the present observations are too few, and the spread too great, to confirm this.

The habit of snapping is associated with defensive and offensive activities. In closing the snapping claw, a vigorous jet of water is produced by means of a plunger arrangement described below. This sudden gush of water serves to frighten away enemies approaching too near. The antagonist may also be driven

away or sometimes killed by a direct blow of the small hammer, but the biological significance of the accompanying loud sound is uncertain.

### The snapping mechanism

A good deal has been written regarding the sound-producing mechanism, especially by Coutière (1899), Brooks and Herrick (1894), and Volz (1938). This mechanism consists of one disproportionately large hard appendage comparable to one of the large "claws" of the lobster. Figure 4 shows an enlarged drawing of the snapping claw of *Crangon californiensis*, a vigorous snapper (cf. Fig. 2).

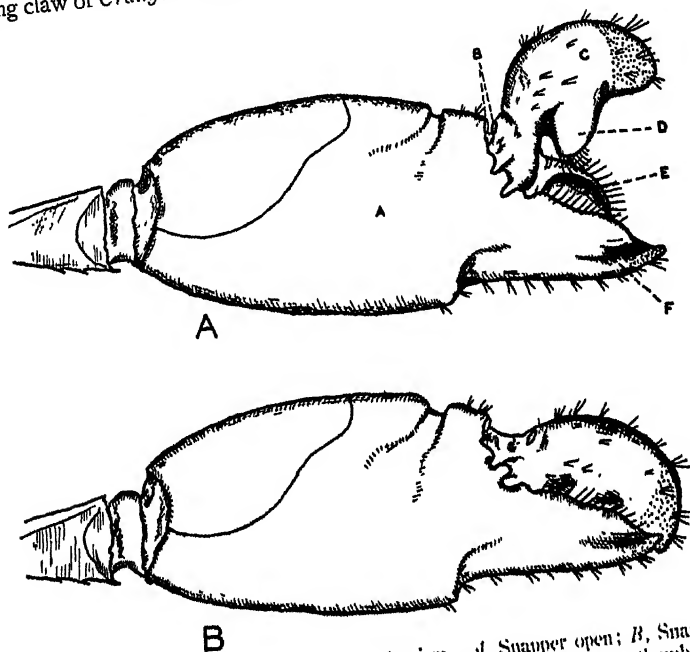


FIGURE 4. *Crangon californiensis*. Snapping mechanism. A, Snapper open; B, Snapper closed. A, palm; B, suckers; C, movable finger; D, plunger; E, socket; F, thumb.

In some species, the snapping claw is nearly as large as the body of the animal, giving ample room for development of strong snapping muscles. A movable hard "finger" (C) is jointed near the outer end of the "palm" (A) of the claw forming a forceps-like arrangement with the immovable "thumb" (F). On the inner side of the finger is a plunger-like structure (D) which fits into a socket (E) in the thumb. Leading forward from the socket is a groove through which the water escapes when the plunger is suddenly forced into the hollow. When the finger is raised, the plunger is withdrawn from the socket, and it has been held by some early observers that the noise is produced by this action, similar to the "pop" heard when a cork is suddenly pulled out of a bottle (Wood-Mason, 1878). The biological function of the plunger-socket arrangement, however, is the production

not of sound, but of the water jet mentioned above. It may aid, also, in preventing dislocation of the finger when it is violently snapped.

The plunger-socket device is present in both *Crangon* and *Synalpheus*. In all species of *Crangon*, there is also a "sucking" disc on the outer side of the finger near its point of attachment (Fig. 4A). When the finger is fully raised, this disc contacts precisely a similar disc on the palm so that the two are firmly in contact when the finger is in position to snap. These suckers serve as a trigger to hold the finger back, and extra muscular tension must be exerted in breaking the contact. This in turn increases the force of the impact. The suckers are absent in *Synalpheus*, but in the same relative position there are smooth surfaces that contact perfectly. These surfaces probably serve the same purpose as the suckers. That the suckers on the claw of *Crangon* do serve as a restraining trigger is readily demonstrated. When the finger of a dead specimen is forced back to its fullest extent, it is held there so firmly that some force is needed to break the contact. This was demonstrated even in a specimen that had been preserved in formaldehyde for several months.

The precise way in which the sound is produced is still a matter of some doubt. The best explanation appears to be that the sound is produced by impact as the movable finger strikes a glancing blow on the opposing tip of the thumb. The tips of these structures are either very heavily calcified as in many species of *Crangon*, or are tipped with a heavy formation of chitin as in *Synalpheus lockingtonii*.

In a painstaking investigation of *Crangon dentipes* and *Synalpheus laevimanus* of the Mediterranean, Volz (1938) found a small chitinous ridge at the inner upper margin and back wall of the socket. This ridge, he concluded, is part of a breaking device which incidentally aids in producing the sound when it is struck by the plunger as it forcibly enters the socket.

It is of passing interest to note that the snapping claw may be either right or left; if it is lost through injury the inconvenience is only temporary, because a new one is promptly grown in the first or second moulting when the shrimp sheds its old shell. In this process of renewal, however, the original large claw is replaced by a small one, while the former small pinching claw is enlarged and becomes the new snapping claw (Wilson, 1903).

#### *Type of habitat and crackling areas*

The species of *Crangon* and *Synalpheus* are bottom-living animals living mainly below zero tide level and, though capable of swimming, they rarely do so in the adult stage. They are notably secretive and demand ready-made or easily maintained burrows. Hence, they seek concealment in crevices and holes provided by coral, stones, shells, calcareous algae, and other solid objects. It has been demonstrated repeatedly by collectors that they live preponderantly on these bottom types. This habit renders collecting very difficult in most instances, especially when a dredge must be used. Hence the animals are far more abundant than generally realized.

In the East Indies they are especially fond of bottoms covered by the calcareous alga *Lithothamnion* (de Man, 1916). They commonly live among the roots of eel grass and holdfasts of seaweeds and among mussels, barnacles, and other substantial

fouling organisms on wharf piles, etc. Some live in the channels and pores of living sponges. In many of these situations there can be heard a continuous fusillade of snapping. Where sound measurements have been made at old heavily-fouled piers, the spectrum level in the region of 3 to 10 kc has reached about 60 db above 0.0002 dyne/cm<sup>2</sup>.

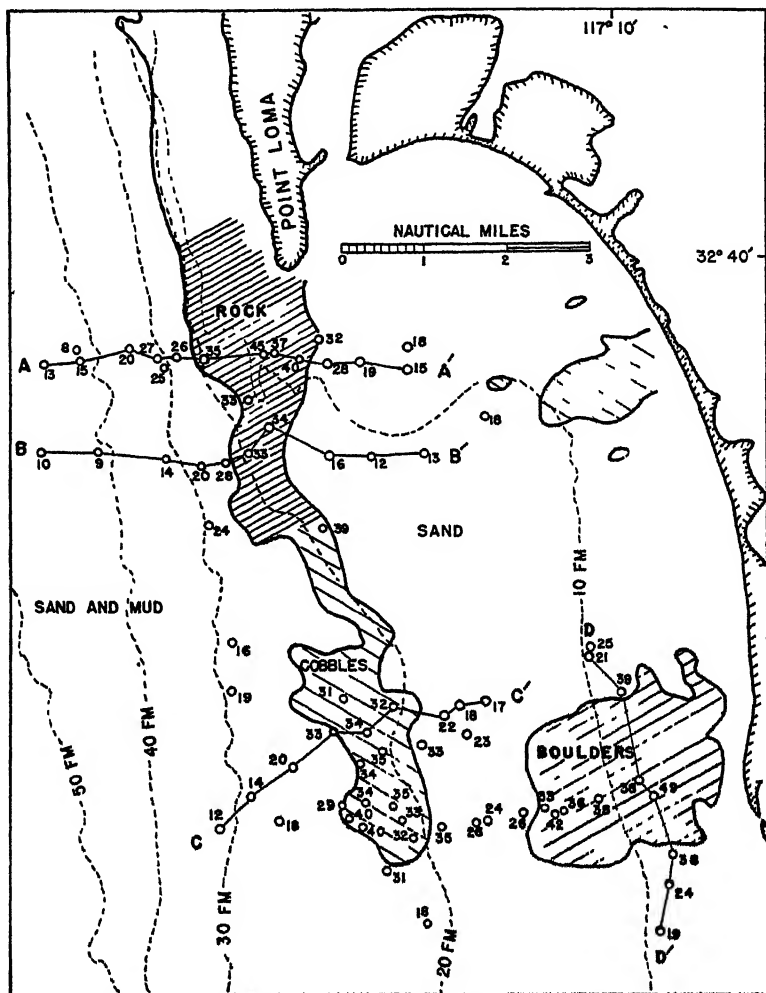


FIGURE 5. Locations of series of stations occupied for sound level measurements across areas ecologically favorable to snapping shrimp. The number adjacent to each station is the average of the 10, 15, and 20 kc spectrum levels.

Snapping shrimp do not normally live in any significant numbers on extensive sand or mud bottoms without some sheltering materials. However, some species (for example, *Crangon californiensis*), tolerate muddy conditions among eel grass, etc., and have been dredged with mud, particularly in harbors littered with solid

debris. Crackling is intensive in some of these situations. A European species, *Crangon ruber*, is said to be "not uncommon" on the mud bottom off Ram's Head near Plymouth, England and in the Mediterranean. No information is available regarding the underwater sound produced by this species.

The way in which the crackling noise increases over a habitat favorable to shrimp is indicated by Figures 5 and 6. Figure 5 shows some of the stations in

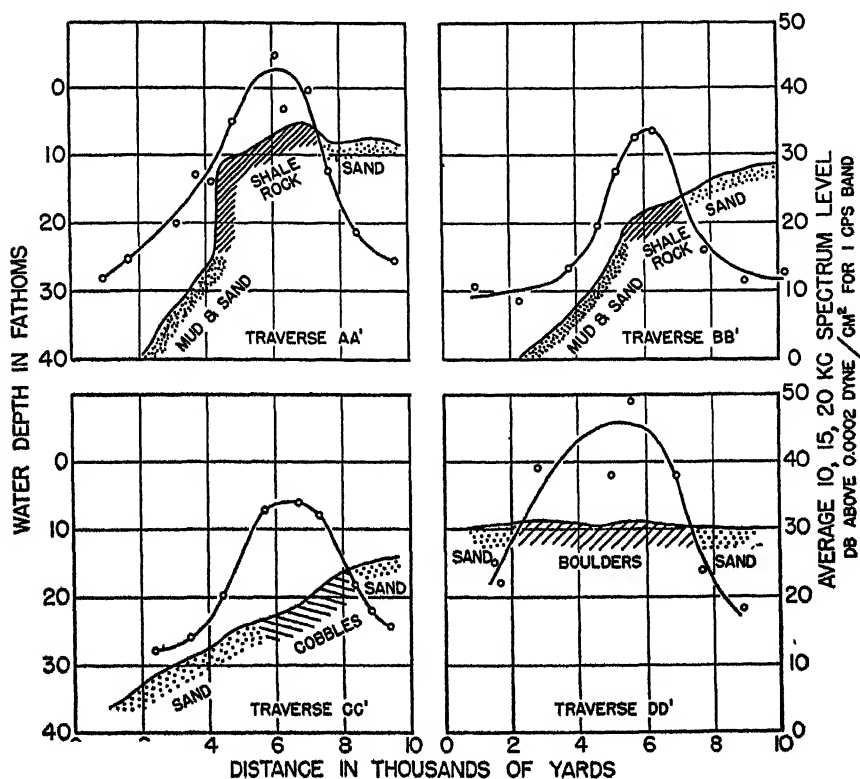


FIGURE 6. Profiles of bottom at stations occupied in Figure 5 showing spectrum levels across shrimp beds.

the San Diego area at which sound spectrum measurements were made under careful control. The number adjacent to each station is the average of the 10, 15, and 20 kc spectrum levels. Water depths may be estimated from the depth contours. The bottom character here is well established.<sup>5</sup> There is a clearly defined stretch of shale which is well honey-combed by abandoned mollusk borings in which the shrimp find retreats. Continuous with the shale is a less regular stretch of cobbles. Nearby is an isolated patch of boulders. Dredgings reveal all these to be populated by shrimp in contrast to dredgings from the surrounding sand-mud and sandy areas of comparable depths.

<sup>5</sup> The assistance of Dr. F. P. Shepard in these studies is gratefully acknowledged.



The sound levels between the cobbles and boulders are higher than would be expected over sand, but this apparently results from a combination of sound transmitted from both areas since the positions are well within the sound range of both.

Specific traverses are indicated by *AA'*, *BB'*, *CC'*, *DD'* in Figure 5. The corresponding depth profiles (use left-hand scale) are given in Figure 6 along with the average 10, 15, 20 kc spectrum level (right-hand scale) at each station.

These show how markedly the sound, in the frequency range 10–20 kc, rises in passing over the shrimp habitats. During low sea states, the transmitted shrimp sound is appreciable to a distance of over a mile from the boundary of the bed.

Similar sound spectrum measurements were made at numerous shallow and deep water stations at widely scattered localities on the east Pacific coast, among the Pacific Islands<sup>6</sup> and along the west Atlantic coast and the Bahamas.<sup>7</sup> A typical set of observations, from six stations off Barbers Point, Oahu Island, is given in Figure 7. The coral, algae, and shell bottom extends into deep water, yet the sound level falls off rapidly in depths greater than 30 fathoms, despite a bottom type favorable to shrimp. At the outermost station the shrimp noise presumably merged with the normal ambient water noise.

An average 10, 15, 20 kc spectrum level of 30 db was arbitrarily chosen as a dividing point for sorting the observations, values greater than this being designated "high" and those less as "low." When the data from both the Pacific and Atlantic were studied with regard to bottom type, it was found that within 0–30 fathoms about 80 per cent of the "high" levels were observed over bottom types favorable to shrimp. In contrast, about 90 per cent of the "low" sound levels (below 30 db) for this depth zone were over unfavorable bottom. In view of the difficulty of establishing precisely the character of the bottom over considerable areas, this correlation is surprisingly good.

### *Depth distribution*

Data on hauls of snapping shrimp, taken at various depths, are scattered widely throughout the biological literature (de Man, 1916). Table I shows how 185

TABLE I

*Distribution of 185 species of Crangon and Synalpheus reported from 5 depth zones*

Bottom depth zone	Number of times shrimp reported	Number of species
0–30 fathoms	418	171
30–70	101	59
70–100	5	4
100–250	14	9
Over 250	7	4

species of *Crangon* and *Synalpheus* have been reported as occurring in five depth zones. The table includes only trawling and dredging collections from the bottom.

<sup>6</sup> Many of these field data were collected by W. E. Loomis of the Naval Ordnance Laboratory during a survey in cooperation with that organization.

<sup>7</sup> These observations were made in cooperation with the Columbia University Division of War Research, Bureau of Ordnance and the Naval Ordnance Laboratory.

While most of the species are reported in 0-30 fathom water, it cannot be concluded that Table I gives the actual distribution, since it is not possible to know precisely how uniform the effort and efficiency of collecting have been in various zones. Presumably, it has been roughly comparable with respect to the number of hauls in the first two zones. This is supported by a summary of 3,483 hauls by various expeditions that have contributed to the study of these animals. Among these expeditions are especially those of the USS "Albatross" (1883-1911), the "Siboga" (1899-1900), the "Percy Sladen" (1905), and the "John Murray" (1933-1934). An analysis shows that 17 per cent and 20 per cent of the hauls were taken respectively in the 0-30 fm. and 30-70 fm. zones. The remainder were in deeper water. Thus the table gives a fair idea of the depth distribution and indicates that the largest populations are in the 0-30 fathom zone.

Acoustic data support this belief, for in water deeper than 30 fathoms the sound levels are low (except where sound is transmitted from nearby shrimp beds), even though the bottom type is favorable for shrimp, as in Figure 7. It is probable

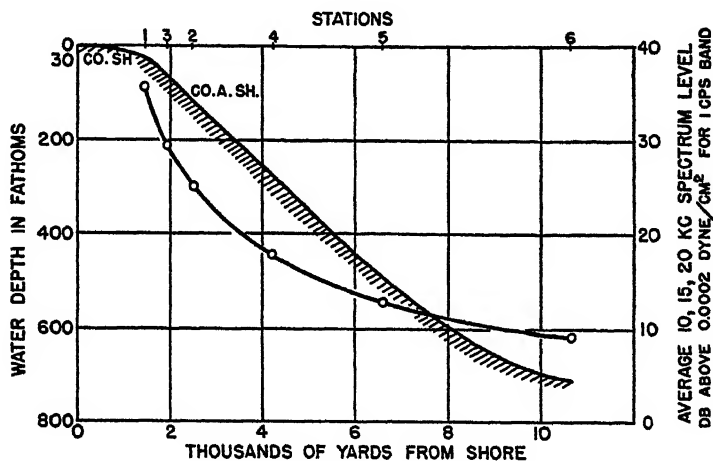


FIGURE 7. Sound observations from Barbers Pt., Oahu showing drop in spectrum level with depth of water and distance from shallow (less than 30 fm.) areas favorable to snapping shrimp.

that the deeper ranging species have their maximum concentration in depths less than 30 fathoms, and that the typically deep water forms do not occur in sufficient concentrations to produce a continuous crackle like that heard in shallow water.

#### *Life history and its bearing on continuity of crackle*

The bedlam of crackling noise over a shrimp bed is never ending. This continuity is correlated with the life history and stability of shrimp populations. The fertilized eggs of snapping shrimp are carried by the adult female until they hatch as free-swimming larvae about 4 mm. in length. After a period of drifting with the water currents, the larvae settle to the bottom and assume the structural characteristics and habits of the adults. No snapping is possible until this time. The

free-floating period serves to disperse the animals widely and they thrive after settling to the bottom where living conditions are favorable for adults. From what is known of a few species, there appears to be a long breeding season in low latitude waters. Gravid specimens have been found in all seasons at La Jolla. Near the higher latitudes the breeding season is probably shorter.

The duration of life of the individual is not known, but adults collected at La Jolla, California, in December 1942, and January 1943, were still thriving in an aquarium after twenty-two months of captivity. These facts are mentioned because the maintenance of overlapping generations is, no doubt, important in explaining the seasonal continuity of the crackling sound. A short breeding season and a short span of life would result in a considerable variation of the adult population and this, in turn, would be expected to cause an appreciable seasonal variation in shrimp noises. No seasonal variation of this nature has yet been noticed.

Observations have not revealed any swarming or migratory movements such as characterize many other marine animals during the breeding season, and this, too, is correlated with the small fluctuations in sound level.

The snapping shrimp are gregarious to the extent that large numbers of a given species are found either in pairs or as solitary specimens in isolated retreats within the area. The occupants of these retreats make a noisy protest when an intruder approaches. Specimens have been observed to leave their burrows for short journeys in search of food, especially at night, but those under observation always returned to their respective retreats. These retreats in some instances were occupied steadily by the same specimen for several months. *Crangon californiensis* showed a much stronger tendency to desert its burrow than did other local species observed.

There is a small diurnal variation in shrimp noise. At night the levels are 2 to 5 db higher than in the daytime. In addition, there is a slight peak in the noise level shortly before sunrise and after sunset. The effect is caused, probably, by increased activity of the shrimp at these times. In the laboratory tanks the shrimp appear to increase their search for food at night. This is also a well-known behavior among many other marine animals. Thus, the overall increased activity would lead to more irritation and more snapping.

It should be noted that an increase in sound level of 3 db actually represents a two-fold increase in shrimp activity.

Observations made so far have indicated no appreciable seasonal variation in shrimp noises. This is in agreement with the known stability of the adult population discussed above. This result may not apply near the edge of the shrimp belt, however, or at unusual localities inside the belt having large seasonal variations in water temperature.

#### *Geographical range of shrimp correlated with distribution of crackle*

The species of *Crangon* and *Synalpheus* are confined to coastal or shallow water throughout the tropical and subtropical regions. This type of distribution is, no doubt, governed by water temperature, but precisely how this operates is not yet clear. In general, the 11° C. winter surface isotherm marks the approximate northern and southern limits of their continuous range (Fig. 8). In Figure

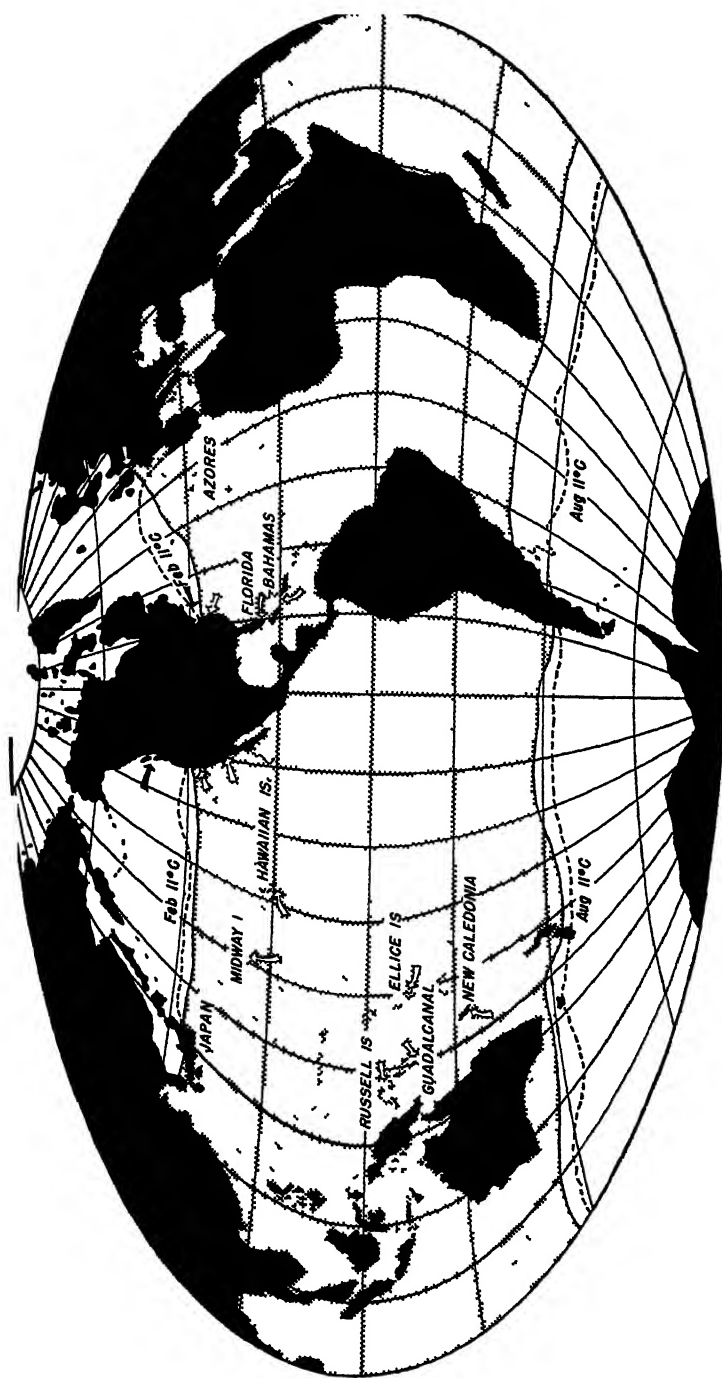


FIGURE 8. Geographical range of snapping shrimp (*Cangon* and *Synalpheus*) These genera are found within the shaded area where depth and bottom conditions are favorable The arrows denote general areas where ambient noise has been measured.

8 this range is represented by the shaded area. The thermal control of distribution must, however, be a complex one. It is not accomplished simply by the temperatures falling to or below this level during the winter. Certain critical periods of life such as spawning and larval development appear to require a considerable period of warmer temperatures, near or above 17° C. during the summer. The isolated populations lying outside of the 11° C. isotherms are probably governed by favorable local conditions. Examples of this sort are seen at the north end of Honshu, near Vladivostok, and along the coast of Ireland. A comprehensive study of the literature giving the localities where snapping shrimp have been collected one or more times, shows them to be present in all tropical and subtropical locations where extensive surveys for crustacea have been made. In the strictly tropical and subtropical waters, the distribution of shrimp is believed, therefore, to be continuous within the areas where depth and type of bottom are favorable. This agrees with the available acoustic data from sound surveys made in various parts of the world.

There is but little quantitative information on geographical distribution. The animals are, as a rule, difficult to collect in representative numbers because of their habits of seeking retreats in hollows and crannies of rock, coral, etc. It seems clear, however, that the greatest number of individuals as well as species occur in tropical waters among coral and other reef organisms. The northern and southern boundaries of continuous distribution along the open coast are, no doubt, transition zones in which the populations become progressively sparser, but how wide these zones are is not known. However, the animals are known to be quite abundant at Beaufort, N. C. and at Monterey Bay, California, and listening tests in these areas reveal shrimp crackle to be pronounced, though spectrum measurements are not available. The region between Cape Lookout and Wimble Shoals on the east coast and the region of San Francisco on the west coast represent the border of distribution (Hay and Shore, 1916; Schmitt, 1921). Sparse crackle has been heard in these regions, but sound measurements fail to show the characteristic spectra of shrimp noises. Acoustically, there must be a certain minimum concentration of shrimp in a given area before the snapping sound can materially influence the reading of instruments which require an appreciable time to respond. Sound measurements from latitude 9° S. to 33° N. indicate about the same levels over shrimp beds irrespective of latitude.

#### BIOLOGICAL SOUNDS SIMILAR TO SHRIMP NOISE

Among the known animals producing noises that may be confused with shrimp crackle or that may in a minor way contribute to the ambient noise are the following:

*Gonodactylus oerstedii* (and no doubt other species of this genus), a crustacean generally known as "mantis shrimp" or "squillid," makes a clicking sound when striking out with its claws. Like the snapping shrimp, this animal has a wide tropical distribution and lives in similar habitats.

At least two species of the *Coralliocars* shrimp, *C. graminea* and *C. wilsoni*, are capable of snapping by means of a structure similar to that used by regular snapping shrimp. They are not known to be abundant and apparently have a geographic and habitat range falling within that of the snapping shrimp.

*Typton spongicola* of the Mediterranean and *Pontonia pinnae* of East Africa are species of crustacea said to be capable of snapping. They are not considered numerous.

The larger crabs such as *Cancer* and *Portunus* have been observed to make noises sounding like the individual crack produced by *Crangon* and *Synalpheus*, but the noise is incidental to the cracking of brittle shells of small clams, etc., for the food within the shell. Crackling from this source can, of course, be present only as long as shell food is being eaten. Other animals, including fishes, which occasionally crack shells for food would also fall into this category of incidental noise makers. To produce the volume of crackling that occurs continuously over shrimp beds would, however, very quickly exhaust all available shells. Hence cracking of shells is believed to be a very small contribution to the ambient noise.

The trigger fish is said to be capable of making a clicking sound by means of the joints of some of the fin spines; the mackerel by means of its pharyngeal teeth.

Populations of barnacles produce very weak crackling sounds, barely audible at very close range. Barnacles and perhaps other larger crustacea (preening their shells or feeding) appear to be the main cause of these very faint noises sometimes heard when the hydrophone is within a few feet of the shore in quiet waters north of the geographic range of snapping shrimp.

In the Hawaiian area, a very troublesome raucous noise with a sharp high peak at 3 kc has been encountered. This has been called "evening noise" in view of its regular occurrence each evening between about 7:30 and 10:00 P.M. In one harbor, this caused a 10 db rise in overall level of the ambient noise during the evening hours. The origin of the noise was not determined, but the possibility of its being somehow associated with increased shrimp activity at a distant bed in the evening has been suggested. However, an obvious difference in character between shrimp noise and "evening noise" militates against this explanation.

### CONCLUSIONS

1. The sea is far more noisy, as a result of biological noises, than was commonly believed.
2. The most widespread of the biological underwater sounds is a continuous high frequency crackle caused by snapping shrimp (*Crangon* and *Synalpheus*). The continuity of high magnitude sound produced by populations of these animals appears to have no counterpart in biological nature.
3. All shallow water areas (0-30 fathoms) within the geographical range of *Crangon* and *Synalpheus* and with bottom conditions and temperatures favorable to these animals are likely to be noisy.
4. The strongest components of shrimp noise are in the frequency range 2 to 15 kc.
5. The sound levels over snapping shrimp beds are usually 30 db higher than state 1 sea noise.

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# BEHAVIOR OF THE CELL SURFACE DURING CLEAVAGE. VII. ON THE DIVISION MECHANISM OF CELLS WITH EXCENTRIC NUCLEI

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The conventional diagram of a typical cell shows the nucleus in a central location, but actually in many cases the nucleus does not occupy the exact geometrical center of the cell. The present paper deals with the mode of division of cells with excentric nuclei, especially in connection with the general theory of the mechanism of cell division proposed in a previous paper of this series (Dan, K., 1943b).

## REVIEW OF THE PROPOSED THEORY

The salient features of the theory are as follows: (1) The asters of sea-urchin eggs are spiny spheres with numerous gel rays radiating out in all directions. (2) Cleavage is brought about as a result of the pushing apart of two such radiate spheres by the autonomous elongation of the spindle (Fig. 1). Applying this concept, a cell in the resting stage can be regarded as a mass of fluid cytoplasm contained in a gelled cortex. When two radiate gel spheres which have developed and reached their maximal size within this fluid cytoplasm are pushed apart by the spindle, the original fluid mass will be divided into two portions which are surrounded externally by the cortex and supported internally by the spiny spheres (Fig. 1). Observations on adhering kaolin particles have shown that the surface of the furrow expands during the latter half of the division process (Dan, Yanagita and Sugiyama, 1937; Dan, Dan and Yanagita, 1938). We can account for this expansion phase if we visualize a definite amount of fluid cytoplasm being divided by the above mechanism. As the asters are pushed apart, the cortical layer covering the cleavage furrow will be sucked in between them and will show a linear stretching along the sides of the furrow. But actual observations have also shown that, at the beginning of the cleavage process, before the expansion phase, the furrow surface undergoes a phase of shrinkage. This initial shrinkage phase may be explained by two more details of the division process. (3) The astral rays cross at the future cleavage plane (Fig. 1, Stage 2), and (4) the rays are anchored to the cortex of the cell. As was fully discussed in the previous paper (Dan, K., 1943b), the rays exert a traction effect upon the cortex of the egg and this effect causes the initial shrinkage, whereas the later expansion of the furrow surface is due to the suction effect mentioned above. Let us consider this more fully. If the underlying spindle elongates while the tips of the individual rays are attaching to the cortex, the loci along which the tips of the crossing rays will move are practically the same as the loci of the apices of triangles made by the crossing rays, the spindle (i.e., the base of the triangle) and a line connecting the tip of each ray with the astral center of the opposite blastomere. Such loci form a curve directed toward the base line and gradually approach-



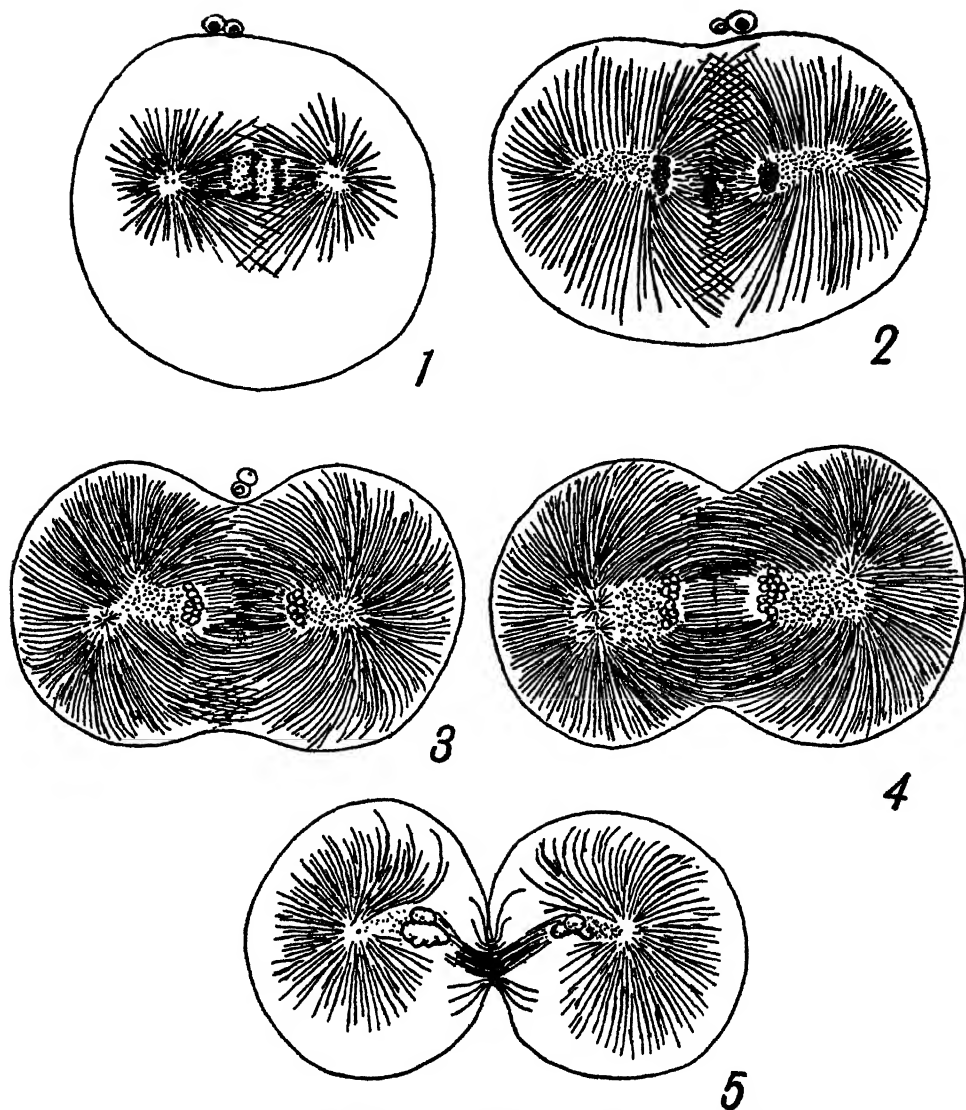


FIGURE 1. Division of the eggs of *Cerebratulus lacteus*. Reproduction of Yatsu's Text Figure C. Legends from Yatsu. "Five stages of the first cleavage,  $\times 400$ . 1. Anaphase of the first cleavage mitosis. Crossing of rays, and the position of the spindle. Difference in distance between two centrioles at either end. 2. Telophase of the first cleavage mitosis. The centrosome has enlarged and been bent downwards. The crossing of rays has begun to loosen. A faint indication of fountain figure is seen at the pole regions. 3. Telophase; a vertical section through the poles and a centriole. Constriction has begun on both the animal and vegetative sides. Sheath spindle has been formed. Fountain figures at the poles of the spindle have become more distinct. 4. Telophase, a horizontal section through three centrioles (about the same stage as 3). Sheath spindle is very well formed. Centrosome has greatly enlarged. The distance between the rows of karyomeres is approximately the same as the original length of the spindle. 5. Late telophase (a vertical section). Sheath rays have been formed. Fountain figure in the equatorial rays."

ing a vertical line bisecting the base (Fig. 2). As the triangles become less equilateral the curve becomes greater. On the contrary, if two astral rays meet exactly on the median plane an equilateral triangle is obtained and the locus of the apex coincides with the vertical line bisecting the base. Seven such loci are shown in Figure 2. As an example, if a pair of symmetrically crossing rays is considered, two unequilateral triangles which are mutually mirror images are obtained and their apices will converge toward the mid-line as the spindle elongates. This means that the surface included between the apices will be made to shrink. The situation is

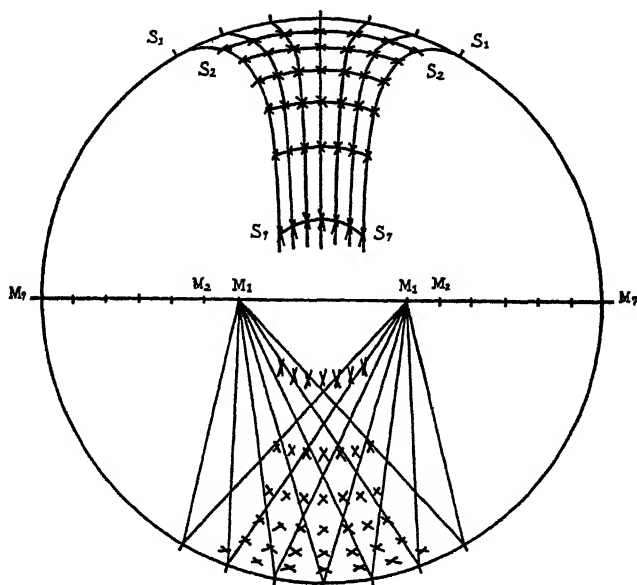


FIGURE 2. Loci of seven pairs of rays which meet end to end and which are attached to the cortex when the spindle elongates. The lower half of the figure shows the positions of the tips of the rays for seven different lengths of the spindle such as are indicated on the horizontal line. The upper half shows the same groups of points in terms of the surface. Notice that the rate of shrinkage of the surface is fast in the beginning and slow later, while the rate of the inward shift of the surface is slow at the beginning and fast toward the end. Note also that the wider the range of crossing of the rays, the greater is the rate of shrinkage. The innermost position of the surface ( $S_7S_7$ ) indicates that a complete division of the cell cannot be achieved by this mechanism alone.

most clearly demonstrated by an experimental model, shown in Figure 3. Since both the traction effect of the astral rays and the suction effect in the cortical layer of the furrow are equally direct results of spindle elongation, there is no contradiction of the statement made under (2).

Finally it must be considered how and when the traction effect is relayed over to the suction effect. To explain this another feature of the division figure must be examined. (5) A fountain-figured bending of the polar rays takes place (Fig. 1, Stages 3 and 4). If the rays were rigid and not pliable, as soon as the spindle elongated the suction effect would be felt at the cleavage plane and there would be

no time for the traction effect to play its role. But actually the rays are pliable. Therefore when the spindle elongates and pushes the asters against the polar surfaces, the rays yield to the pressure and bend in a fountain figure. By this bending of the rays, the spindle elongation is not transmitted directly to the cell contour but is buffered. Once the bending of the polar rays reaches the maximal degree, however, further lengthening of the spindle brings about the suction effect. Thus, even though the dynamic cause of cell division is single—namely, the spindle elongation—its effect on the cell surface involves two factors which come into play in succes-

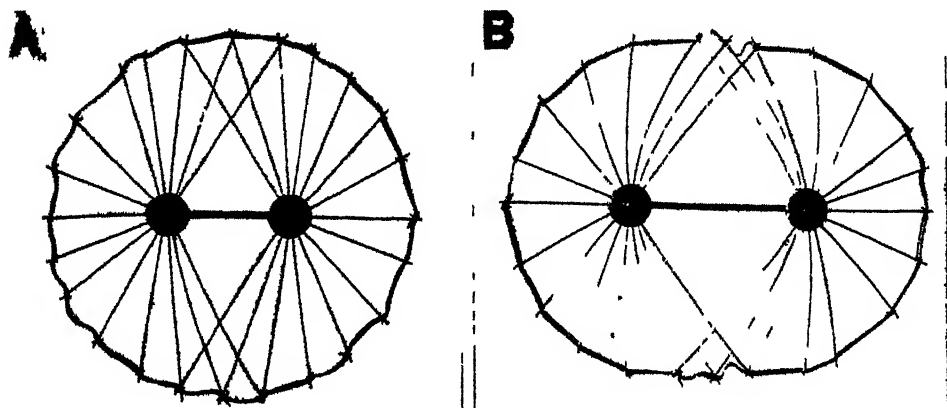


FIGURE 3. A model imitating the initial shrinkage phase of a cell with a central spindle. Astral centers are represented by wooden wheels in which bamboo rods are firmly planted imitating astral rays, which, in turn, are tied to a rubber band simulating the cell membrane. A wooden bar which represents the spindle is also firmly fixed to each wheel, the bars from the two wheels overlapping in the spherical condition (A). The bars have small holes along their lengths so that they can be fixed in any desired length by putting a pin through these holes (B). In B, note (1) the flattening of the furrow regions, (2) the shrinkage of the furrow surface, (3) fountain-figured bending of the polar rays and (4) consequent stretching of the polar surface. Note also that (5) the spindle is straight after the elongation.

sion. It may be pointed out in passing that the fountain-figured bending of the polar rays theoretically ought to bring about an expansion of the overlying surface (polar surface). As a matter of fact, the polar surface does expand from the beginning of the cleavage process, in contradistinction to the furrow surface.

#### THEORETICAL CONSIDERATION OF THE DIVISION MECHANISM OF CELLS WITH EXCENTRIC NUCLEI

Returning to the division of cells with excentric nuclei, one might say that there are only two known facts which are widely accepted today. One is that the mitotic figures resulting from excentric nuclei are also situated excentrically in the cells. The other is that the cleavage furrow appears first at the side of the cell nearest to the excentric mitotic figure, later spreads around the two sides and finally completes its circuit at the opposite side of the cell from that at which it was first formed (Ziegler, 1898).

In order to repeat the observations, the authors used the eggs of the sand-dollar, *Astriclypeus manni*. In these eggs the mitotic figures are excentric toward the animal pole, and the furrow starts to appear from there. The astral rays are much better defined than in other forms. They are clearly seen crossing all along the median plane despite the fact that the rays going to the animal hemisphere are shorter than those distributed in the vegetal hemisphere. When the cell bodies begin to elongate for cleavage, the polar fountain figures of the rays are also seen quite definitely. Let us see if these division features of an excentric egg can be harmonized with the concepts of the proposed theory.

In the first place it might be anticipated that the furrow would appear earlier at the side nearer the mitotic figure (the animal pole in *Astriclypeus*), because, during the initial stage of cleavage when the rays are crossing, for a given elongation of the spindle the tips of the shorter rays would be pulled in more than those of the longer rays. Later, after the crossing of the rays is dissociated, a wider gap would form between the parts of the asters with shorter rays than between those with longer rays, resulting in a deeper furrow in the region of the shorter rays. So far the concepts are quite adequate, although they were originated to explain the division of eggs with centrally located nuclei. Let us repeat them: The asters are spiny gel spheres and cleavage is the consequence of their separation by the elongation of the spindle.

There is, however, a contradiction. For if an excentric spindle simply elongates in the ordinary fashion, even though the furrow forms earlier on the side where the rays are shorter, by the time cleavage is nearly completed the blastomeres ought to assume the shape shown in Figure 4. Since this never happens in actual cleavage, some other factor must be operating.

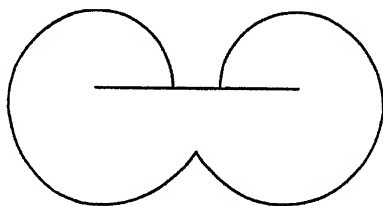


FIGURE 4. The probable shape of the blastomeres if an excentric spindle should simply elongate without bending.

Perhaps another model, this time one with the spindle and astral centers located above the middle of the egg, will help to explain this factor (Fig. 5, *A*). If the asters are pushed apart and their positions fixed by putting a pin through the holes in the bars representing the spindle, these automatically form an angle (Fig. 5, *B*). Since no special rotating force is being applied under this circumstance, the flexion of the "spindle" must be the direct result of the simple separation of the "asters." If this model experiment conveys a correct picture, it leads directly to the suggestion that the spindle may be bending during the cleavage of *Astriclypeus* eggs.

In the model, the mechanical force causing the flexion of the bars is very easy to understand. The tips of the rays (bamboo rods) are attached to the cortex (rubber band). Therefore, when the astral centers are pushed away from each

other by a given distance, the tips of the rays are left behind by that distance in relation to the astral centers. Under such a circumstance, the shorter rays have to rotate through larger angles than the longer rays in order to cover the shifted distance. This difference between the rotating angles of the rays of the two sides will work, in the net result, so as to rotate the whole aster in the direction of the shorter rays, provided that the aster is rigid and can maintain a constant shape. In the model, these rotations naturally force the two bars, each attaching to one "aster," to make an angle between them.

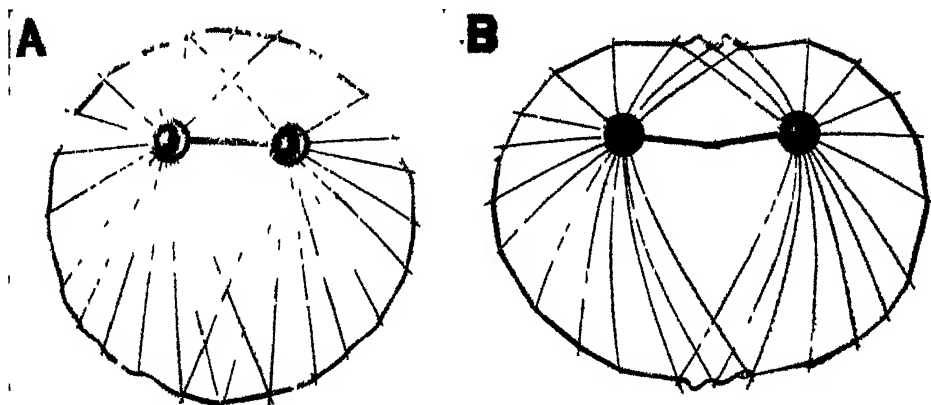


FIGURE 5. A model similar to that shown in Figure 3, except that the spindle and the astral centers are made excentric (A). In B, after separating the astral centers and fixing their positions by putting a pin through the holes in the bars, note the following points: (1) The fountain figure of the polar rays is obscured and the majority of the rays turn in the same direction in a vortex configuration (compare with Fig. 3B). (2) The innermost pair of the crossing rays at the upper pole is practically straight while that of the lower pole is clearly bending and finally (3) the spindle is flexed in the middle.

The model, however, still cannot imitate the expansion phase, because, being two-dimensional, it cannot take care of the factor of the constancy of the cell volume. But, *a priori*, since the furrow surface is sucked into the gap between the two asters in the expansion stage, the rotation effect will be more emphasized: viz., during the expansion phase of actual cell cleavage, the spindle will bend more acutely, to such an extent that it will even tend to tear open the vegetal region, forming the furrow. It must be pointed out here that in order to allow the free rotation of the two asters around their excentric centers, the volume of the cells would have to be increased, as can be judged from Figure 6. If the volume of the cell were kept constant, the rotation of absolutely rigid asters would be impossible. The fountain-figured bending of the polar rays is also quite significant in this connection, as it makes possible astral rotation within cytoplasm of a constant volume.

#### SPINDLE BENDING IN *ASTRICLYPEUS* EGGS

The next step was to determine whether the spindle is actually bending in the *Astriclypeus* eggs. Even a hasty examination was enough to confirm this fact.

On further investigation, it came to the authors' notice that though the degree of excentricity of the mitotic figure is fairly constant within a single batch of eggs, it varies somewhat among different batches, and the acuteness of the spindle bending is proportional to the degree of its excentricity. In speaking about the degree of excentricity, in order that the statement be accurate, the eggs must be observed strictly from the side.<sup>1</sup> Naturally, the cases with extremely excentric spindles are most convenient for analysis. In Figure 7 are given tracings of micro-photographs showing the mode of cleavage and the conditions of ray bending. Purposely, in the first and the last drawings of Figure 7, eggs with less excentric spindles are shown, while the second to the sixth are successive stages in the cleavage of a single egg with an extremely excentric spindle. In diagrammatization, however, the results of many other observations were taken into consideration.

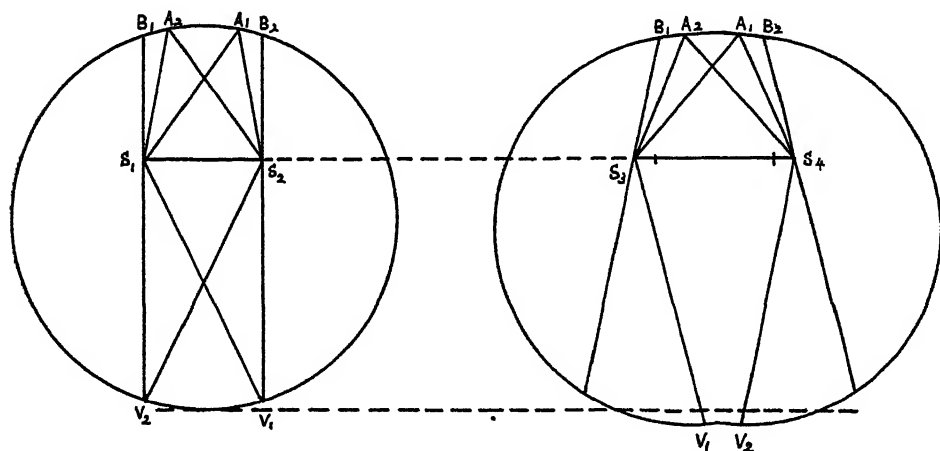


FIGURE 6. The hypothetical result of the simple rotation of asters around their excentric centers, as the result of the shrinkage of the upper pole. Notice the opening of the lower side accompanied by the stretching of the surface there and the bulging of the lower contour. In the actual cell division, such a perfectly free rotation is prevented because the cell volume is kept constant. But the rotation is allowed within a limit corresponding to the degree of bending of the polar rays.

In the first drawing, as far as could be judged from observation on living material and photographs, the astral rays and the spindle are both straight. (Remember that this is a case with less excentric spindle.) Theoretically, in this stage, the rotation of the aster must have begun, but this could not be ascertained, presumably because of its slowness. In the second drawing (an extreme case), the animal furrow is decidedly in the expansion phase. At this stage, two fan-shaped areas appear, one on each side of the cleavage furrow, in which the rays seem to be fewer than in other parts so that these regions are slightly more transparent and

<sup>1</sup> In order to obtain a strict side view of an egg, one takes advantage of the fact that the spindle excentricity is exceedingly constant within one batch. On surveying a culture of eggs with a medium magnification, one can easily get the idea of the maximum excentricity of the spindle as far as that particular batch is concerned. Such maximal cases must represent a full side view of the egg.

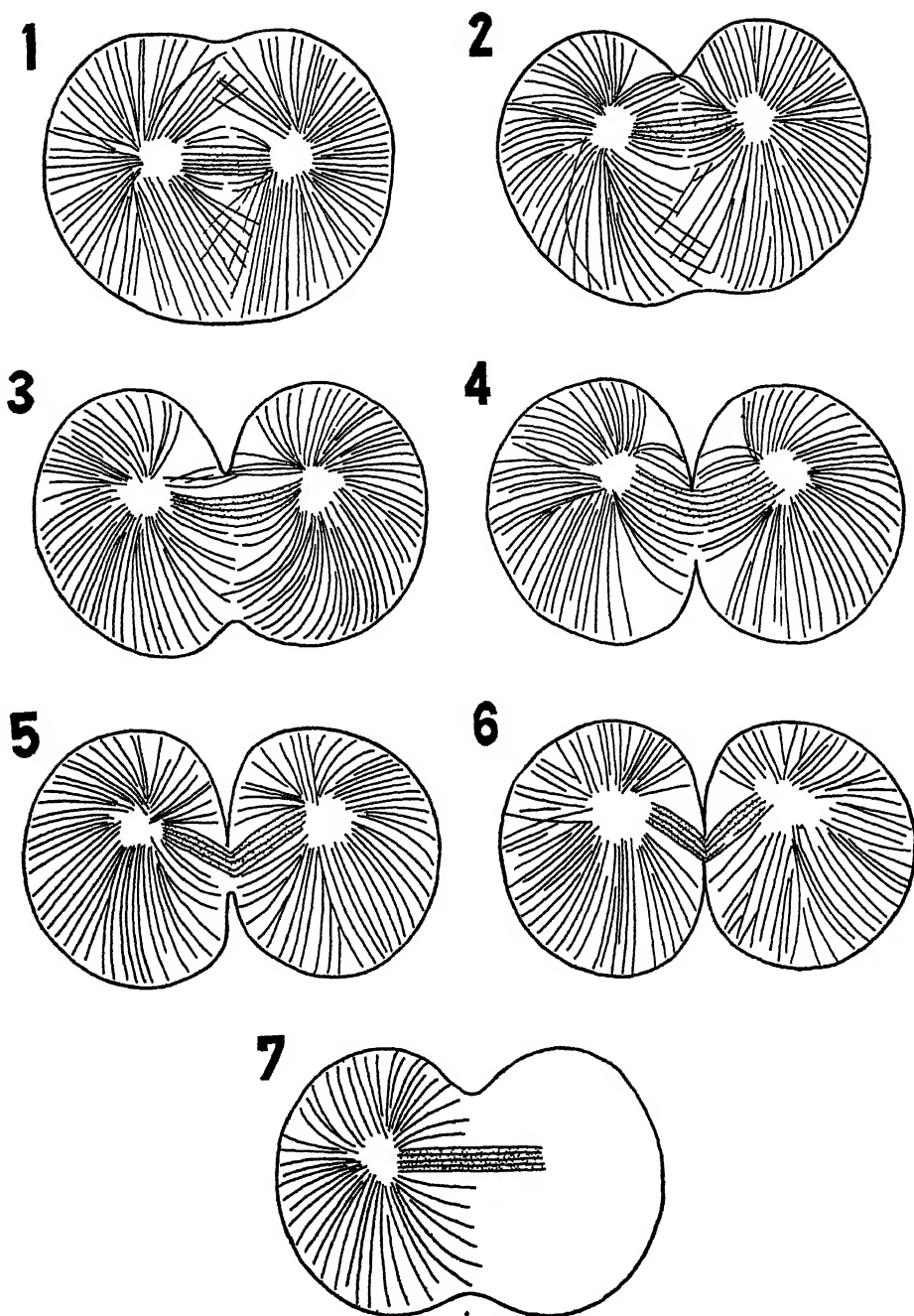


FIGURE 7. Tracings of micro-photographs of dividing *Astriclypeus* eggs showing the changes of curvature of the astral rays in successive stages. The first drawing is made from an egg of a different source but the second to the sixth drawings belong to a series of photographs of a

relatively bright. Since the authors were at first skeptical about this fact, special attention was paid to this point, but numerous observations only served to confirm it. According to the authors' interpretation, the appearance of the fan-shaped area is probably due to the fact that as the furrow begins to be drawn in by the suction effect, the formerly crossing rays are caught by the furrow constriction. Then in the fan-shaped portion just behind the furrow tip, the rays will become relatively sparse. This bundling of the equatorial rays by the furrow must certainly be the beginning of the formation of the so-called sheath rays, described by Yatsu in *Cerebratulus* eggs: "One striking feature of this stage is the formation of the sheath rays around the spindle, due to the fusion of the equatorial rays and a part of the intermediate rays. The spindle shaped sheath rays seem to occur in a good many forms" (Yatsu, 1909, p. 387). Simultaneously with the appearance of the fan-shaped area, the bending of the spindle becomes noticeable.

At the same time, two other changes are taking place in the curvature of the astral rays. One is the bending of the vegetal crossing rays. Among regularly dividing cells, the rays are straight while they are crossing, for they are supposed to be exerting traction force. Therefore, the bending of these vegetal crossing rays deserves especial attention (see Fig. 5). The other change is the curvature of the rays lying in the sector between the fan-shaped area and the spindle polar region. These rays, which have been either straight or bending in a fountain figure (see the right blastomere of the second drawing), now start to bend in unison into the same direction as the vegetal crossing rays, so that the rays of the whole aster display a beautiful vortical configuration (see the left blastomere of the second drawing). These changes in the way of bending of the rays can best be explained by thinking that the asters have been twisted around their centers. In the third drawing, the sheath ray formation on the animal side, the spindle bending and the vortex figure all advance in degree and the vegetal crossing rays have almost been drawn apart. This drawing is of particular interest in two senses. One is that it shows the vegetal half of the egg actually being torn apart by the twist. The other is that it almost coincides with a drawing of Conklin's of the egg of *Cynthia partita* (Conklin, 1905; Pl. VII, Fig. 100). In the fourth stage, the furrow on the animal side has almost reached the spindle and the sheath rays there begin to show a tendency to return to their original positions. On the vegetal side, two fan-shaped areas can be recognized, indicating that sheath ray formation has begun there also. Moreover, the formerly

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single egg. The spindle of this egg is more excentric than that of the first drawing. The seventh drawing is an example of an egg with a far less excentric spindle. Drawing 1, rays are practically straight. Theoretically the asters must have begun to rotate, but this cannot definitely be ascertained. Drawing 2, note the appearance of the fan-shaped clear areas on both sides of the animal furrow in the expansion phase; sheath ray formation; the bending of the vegetal crossing rays; the slight sign of spindle bending. Drawing 3, further development of the fan-shaped bright portion; advance in the spindle bending and dissociation of the crossing rays of the vegetal side. Note also the vortex configuration of the rays. Drawing 4, approximate contact of the animal furrow with the spindle and the tendency of the rays of the animal side to return to a straight condition. Appearance of the fan-shaped areas on the vegetal side. Drawing 5, last moment of division. Disappearance of the fan-shaped areas and definite bending of the spindle. Drawing 6, interkinetic stage. Bending of the vegetal rays in the opposite direction (divergent bending). Drawing 7, division of a cell with less excentric spindle. Notice usual fountain figure of the rays instead of the vortex configuration and apparent straightness of the spindle.



bent vegetal rays seem to have returned to a straight condition. But judging from the fact that the rays on the animal side still retain the same curvature as in the previous stage, this straightening of the vegetal rays may have been induced by the change in the shape of the aster itself.

In the fifth drawing, the cleavage is almost complete and all the rays, including the former sheath rays, are returning to a straight condition. At this stage, the spindle forms an obtuse angle with its apex directed toward the vegetal pole. The sixth drawing shows the egg in the interkinetic stage. Now the vegetal rays are turning in the opposite direction and new fan-shaped, ray-free areas appear adjacent to the now bent spindle. In Yatsu's last drawing, this kind of bending is seen in the rays of the animal side. This post-cleavage bending is what the senior author called "divergent bending" in the previous paper (Dan, K., 1943b), and it is considered to be an indication of the formation of the new surface (see also Dan and Dan, 1940).

In the seventh drawing, the condition within an egg whose spindle is not so excentric is illustrated. Here no vortex configuration is attained and the seemingly straight spindle and the usual fountain figure of the polar rays are seen as in *Cerebratulus* eggs. In this latter form, the spindle is not so excentric and its bending, consequently, becomes apparent only in much later stages, as can be judged by Yatsu's figure.

These observations on the eggs of *Astriclypeus* with extremely excentric spindles help to elucidate the intrinsic mechanism of spindle bending. It seems almost certain that the causative force of spindle bending is derived from the advancing animal furrow and transmitted by the sheath rays to the astral centers which, in turn, impart it to the spindle. But since the advance of the furrow is, itself, nothing but the result of the pushing apart of the two asters by the elongating spindle, an interesting cycle is seen. Thus it can be said that the causative force of spindle bending comes from the spindle's own elongation.

It must be emphasized that the fundamental situation is not at all different from that of typical cleavage. In both cases, sheath ray formation occurs. But in typical cleavage, since the inward pressure which produces sheath ray formation occurs at both the animal and vegetal sides simultaneously, the two effects cancel each other. In the heart-shaped cleavage, the pressure begins at the animal side and for a short time there is nothing to counteract it on the vegetal side. During this time the elongating spindle will encounter resistance on one side only, and will become concave toward that side.

Two other important facts are patent in these observations. One is that the connection between the aster and the spindle is an unexpectedly firm one. There is a striking correlation between the degree of astral rotation and the degree of spindle bending. If this junction were loose, the aster might rotate more, but the spindle could not be bent at all. The second fact is that the spindle must have a certain degree of rigidity, since it can be bent by applying forces at its two ends. The present data give further, and more decisive, support to the possibility postulated by K. Dan (1943a).

Lastly, it must be pointed out that although the model experiment helped to reveal the mechanism of spindle bending, the spindle bending during the shrinkage phase shown by the model could not be demonstrated within the living cell. In actual observation, the sheath ray formation is always seen as the direct impetus for

spindle bending. Then is it permissible to apply this idea, obtained from a model, to actual cells?

#### THE EFFECT OF ASTRAL ROTATION ON SURFACE BEHAVIOR

In the foregoing sections, it was argued from the model experiment that the cause of astral rotation lies in the difference in length of the astral rays on the two sides of an aster. If this is so, the rotation of the aster must be occurring from the very beginning of the cleavage process. Although the observations of the bending of the rays and of the spindle, such as were reported in the previous section, are quite decisive, they can be applied with certainty only to the expansion phase in which the sheath ray formation becomes evident.

For information on the early part of the cleavage process it was thought that a study of surface behavior might be used to establish some unmistakable evidence of astral rotation. Here the kaolin method and its geometrical analysis were employed again. The purpose of the analysis was to test whether or not the furrow formation is really due to the traction effect of the crossing rays. The procedure was to make a sketch of an egg in the spherical condition on which the positions of the kaolin particles and of the astral centers were recorded. From this sketch, the lengths of the crossing rays could be obtained. Once these lengths were fixed, given the spindle length of each stage, the theoretical positions of the particles could be obtained for later cleavage stages. These theoretical positions of the particles could then be compared with their observed positions. If the authors' postulates be correct two conditions follow: (1) The theoretical positions of the particles ought to fall on the sketched contour of the cleavage furrow because the surface is being pulled in by the rays during the shrinkage phase and (2) the degree of shrinkage in the distance between the two theoretical points ought to agree with that of the observed particle-distance as long as the particle-distance coincides with the real crossing range of the rays. In the previous paper of this series, it was shown that the above propositions satisfactorily hold in actual cases (Dan, K., 1943b; Figs. 6 and 7, Table III). But it must be stressed that even when the second condition fails, the first condition proves to be true as long as the deviation between the actual particle-distance and the crossing range is not too great. This means that the second proposition is a stricter test for the hypothesis than the first one. However, in order to apply the second proposition properly, the painstaking accumulation of many readings is necessary to map out beforehand the actual range of crossing of the rays (see Dan, K., 1943b; Fig. 8). On the contrary, the first proposition, although it is less sensitive, is convenient and quite satisfactory as a qualitative test. When the suction force develops and the crossing rays are pulled apart, the intersection points (apices of the triangles) begin to fall outside the contour of the cleavage furrow. The time at which the intersection points begin to deviate is always simultaneous with the onset of the expansion phase.

Now let us see how the surface of *Astriclypeus* eggs behaves in the kaolin experiment. The method has been used on these eggs previously (Dan, Dan and Yanagita, 1938) but the present observations are especially concerned with eggs containing extremely excentric nuclei. Two sets of the records for the qualitative test are given in Figures 8 and 9 and Table I. As for the animal furrow, in the egg shown in Figure 8, the expansion phase sets in at the fifth stage and at this very

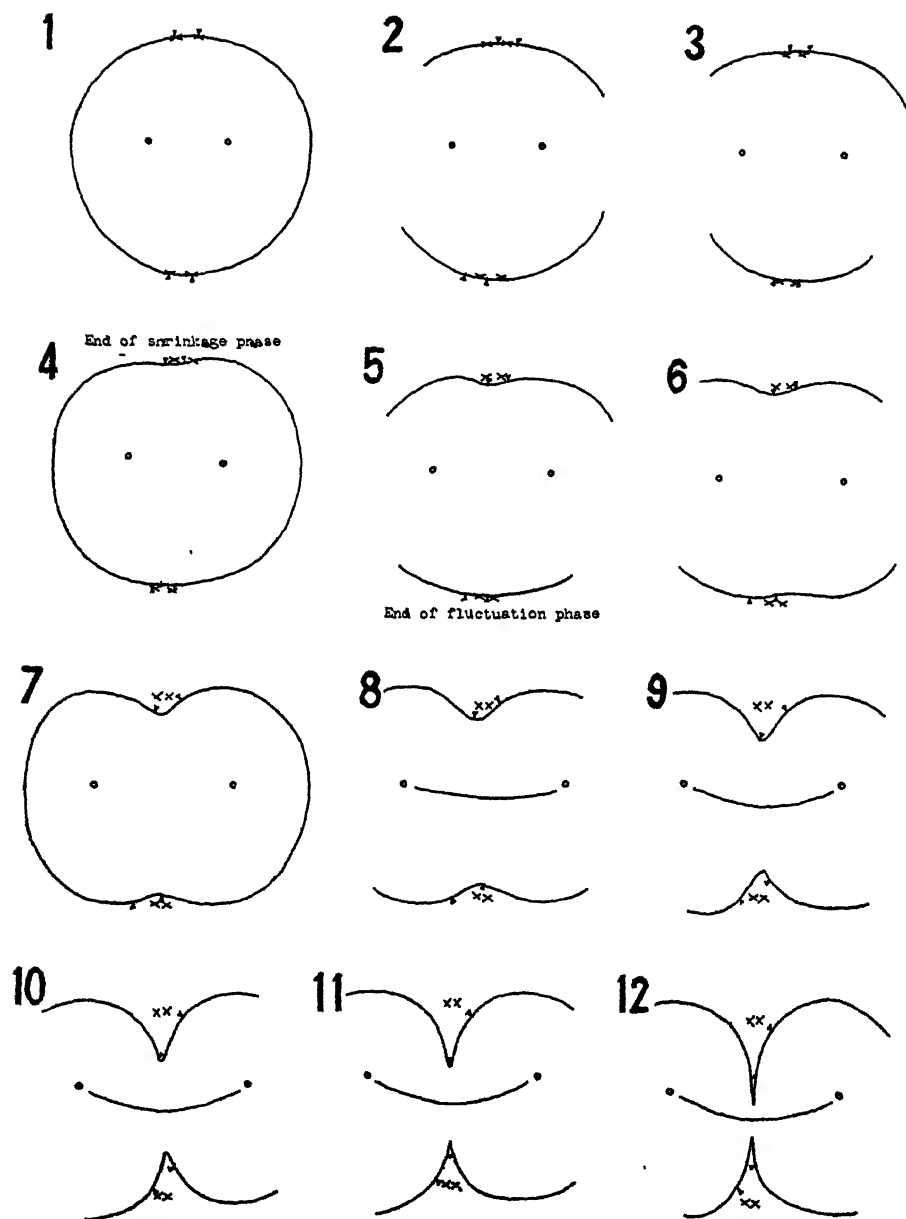


FIGURE 8. Successive camera lucida drawings of a cleaving *Astriclypeus* egg with the recorded positions of kaolin particles ( $\blacktriangledown\blacktriangledown$ ) and astral centers ( $\circ\circ$ ). In the spherical condition (first drawing), the two particles at the animal pole and at the vegetal pole respectively are connected to the astral centers by four hypothetical rays (which, however, are omitted from the figures). In succeeding drawings, by using the sets of four lines obtained in the first drawing as radii and taking the astral centers of successive stages as centers, two intersection points

stage, the intersection points start to deviate from the contour. In the egg shown in Figure 9, this happens at the sixth stage and the coincidence between the onset of the expansion phase and the initiation of the deviation of the intersection points from the contour is again unailing. As far as the vegetal furrow is concerned, in the egg of Figure 8, the particle-distance follows a zig-zag course: 100.0%–97.9%–111.4%–96.9%–104.1%. At the sixth stage, the value jumps to 121.8% which is followed by a further rise. Therefore, the sixth stage can be considered as the beginning of the expansion phase. In the egg of Figure 9, although the course of change is more gradual, the same tendency is unmistakably recognizable: 100.0%–104.0%–103.0%–104.3%–104.6%–100.0%–100.3%. At the eighth stage, the value goes up to 115.2% and the intersection points begin to fall outside the furrow contour. These examples indicate that in eggs with extremely excentric spindles, the initial shrinkage effect is so obscured at the vegetal side that the term "shrinkage phase" is no longer appropriate. For this reason, in the present paper, the term "fluctuation phase" will tentatively be used. But a rather surprising fact is that in Figure 8 the intersection points coincide well with the traced cell contour during this fluctuation phase. In Figure 9, the intersection points do not at least go outside the drawings until the end of the fluctuation phase. From these facts, it seems permissible to conclude that at both poles of the cleaving *Astriclypeus* eggs, fundamentally the same division mechanism is at work.

Then why is this expressed as shrinkage at the animal side and alternate shrinkage and expansion at the vegetal side? It may be due to the rotation of the asters. It was suggested in the foregoing section that when the two asters rotate so that their animal hemispheres converge, the vegetal furrow must tend to be torn open. In reality, the situation may be somewhat more complicated. While the asters turn, if the tips of the crossing rays can retain their attachment to the cortex, the vegetal surface may be made to shrink more than when the rotation is absent. But if these rays pull loose from the cortex and the asters become free to rotate, they may stretch the surface between them on rotation as is diagrammatically shown in Figure 6. If the rays pull loose in several steps shrinkage and expansion will alternate.

Another point which is rather peculiar is the shift of the intersection points to the inside of the cells in the drawings, a form of behavior which has never been met with in regularly dividing cells. For example, in Figure 9, during the fluctuation phase of the vegetal furrow which lasts for seven stages, the intersection points fall inside the cells at two different times including four stages. Stage 3 and stage 5 of Figure 9 are of special interest. In those two stages, the animal furrow being still in the shrinkage phase, the intersection points of that side are coinciding with the contour. As long as the intersection points of the animal side are coinciding with the contour, one is provided with a guarantee that no error is involved in the recording of the positions of the astral centers. Yet the vegetal intersection points fall inside the drawing. This shows that the inward shifting of the vegetal intersection points is not an error but represents something which is actually happening

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(××) are found for each pole. The percentage changes of the observed particle-distance ( $P$ ) and of the theoretical distance between the two intersection points ( $D$ ) are given in Table I together with the time of recording. Note that during the shrinkage phase of the animal pole (from the 1st to the 4th stage) and the fluctuation phase of the vegetal pole (from the 1st to the 5th stage), the intersection points fall on the egg contour.

in *Astriclypeus* eggs. There are two suggestions as to its identity. One is spindle bending. Since the bent spindle is convex toward the vegetal pole, it may push out the contour of the vegetal furrow. The other is that when the asters rotate around their excentric centers, pulling loose their crossing rays, the vegetal contour may bulge out as is indicated in Figure 6. At any rate, the astral rotation may

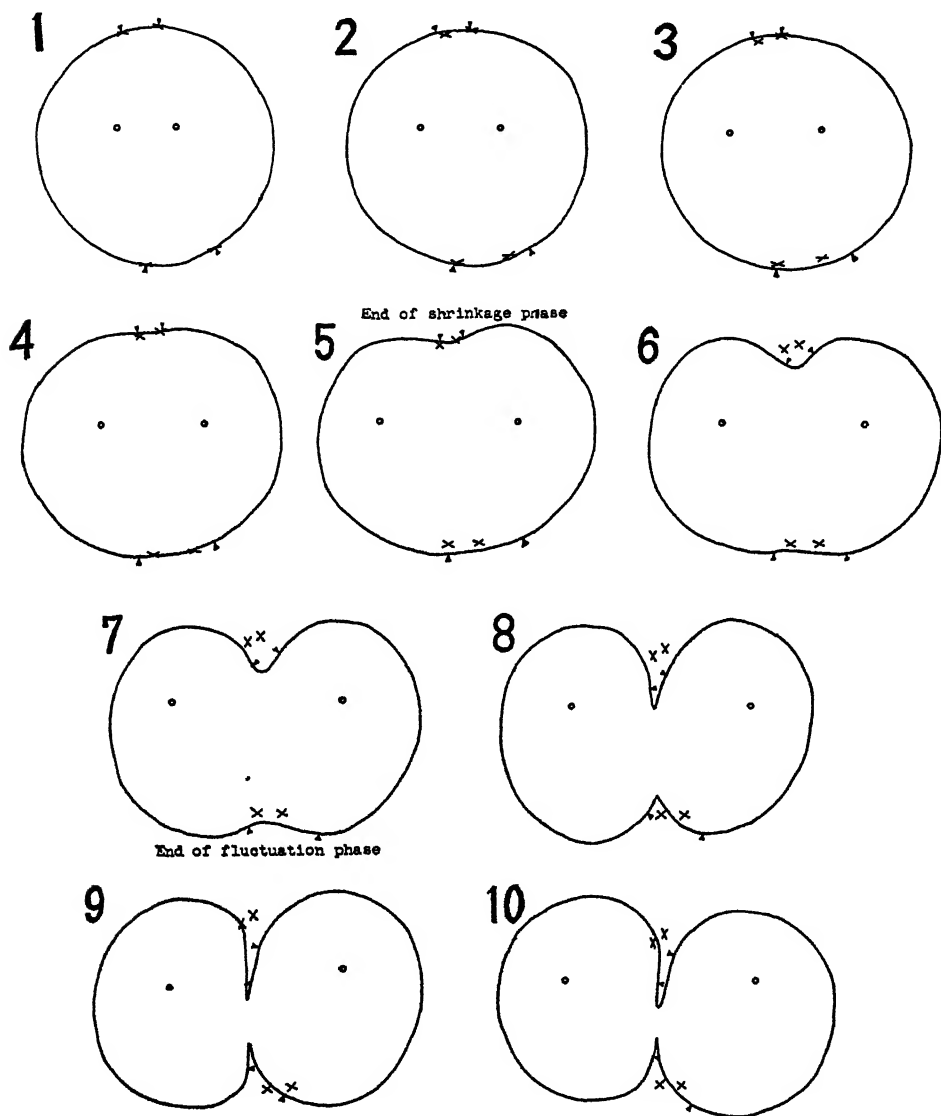


FIGURE 9. Another example of the geometrical analysis of an egg with extremely excentric spindle. The shrinkage stage of the animal pole lasts for five stages and the fluctuation of the vegetal pole ends at the seventh stage. Note that the vegetal intersection points are sometimes inside the contours.

TABLE I

The results of the geometrical analyses of *Astridylpeus* eggs with extremely excentric spindle shown in Figures 8 and 9, exemplifying the "fluctuation phase." *P*, percentage changes of observed particle-distance. *D*, percentage changes of theoretical particle-distance. The case of Figure 8

Stage	1	2	3	4	5	6	7	8	9	10	11	12
Time of recording at 21.0° C.	0"	1'00"	2'00"	3'00"	3'50"	4'40"	5'00"	5'45"	7'40"	8'38"	9'30"	10'35"
Animal furrow	<i>P</i> = 100.0 <i>D</i> = 100.0	<i>P</i> = 93.7 <i>D</i> = 88.5	<i>P</i> = 92.7 <i>D</i> = 75.0	<i>P</i> = 83.3 <i>D</i> = 83.3	<i>P</i> = 97.9 <i>D</i> = 65.6	<i>P</i> = 119.7 <i>D</i> = 61.4	—	—	—	—	—	—
Vegetal furrow	<i>P</i> = 100.0 <i>D</i> = 100.0	<i>P</i> = 97.9 <i>D</i> = 91.6	<i>P</i> = 111.4 <i>D</i> = 90.6	<i>P</i> = 96.9 <i>D</i> = 85.4	<i>P</i> = 104.1(?) <i>D</i> = 69.7	<i>P</i> = 121.8 <i>D</i> = 63.5	<i>P</i> = 127.0 <i>D</i> = 61.4	—	—	—	—	—

The case of Figure 9

Stage	1	2	3	4	5	6	7	8	9	10
Time of recording at 26.5° C.	0"	2'00"	2'50"	4'05"	5'00"	7'00"	8'05"	9'30"	10'45"	12'30"
Animal furrow	<i>P</i> = 100.0 <i>D</i> = 100.0	<i>P</i> = 94.0 <i>D</i> = 73.5	<i>P</i> = 80.8 <i>D</i> = 68.8	<i>P</i> = 72.8 <i>D</i> = 59.6	<i>P</i> = 66.9 <i>D</i> = 47.0	<i>P</i> = 99.3 <i>D</i> = 43.0	<i>P</i> = 114.5 <i>D</i> = 39.1	<i>P</i> = 158.9 <i>D</i> = 39.7	<i>P</i> = 185.4 <i>D</i> = 39.7	<i>P</i> = 218.5 <i>D</i> = 39.7
Vegetal furrow	<i>P</i> = 100.0 <i>D</i> = 100.0	<i>P</i> = 104.0 <i>D</i> = 73.3	<i>P</i> = 103.0 <i>D</i> = 63.0	<i>P</i> = 104.3 <i>D</i> = 55.4	<i>P</i> = 104.6 <i>D</i> = 45.5	<i>P</i> = 100.0 <i>D</i> = 39.3	<i>P</i> = 100.3(?) <i>D</i> = 33.0	<i>P</i> = 115.2 <i>D</i> = 31.7	<i>P</i> = 122.1 <i>D</i> = 33.0	<i>P</i> = 123.8 <i>D</i> = 30.4

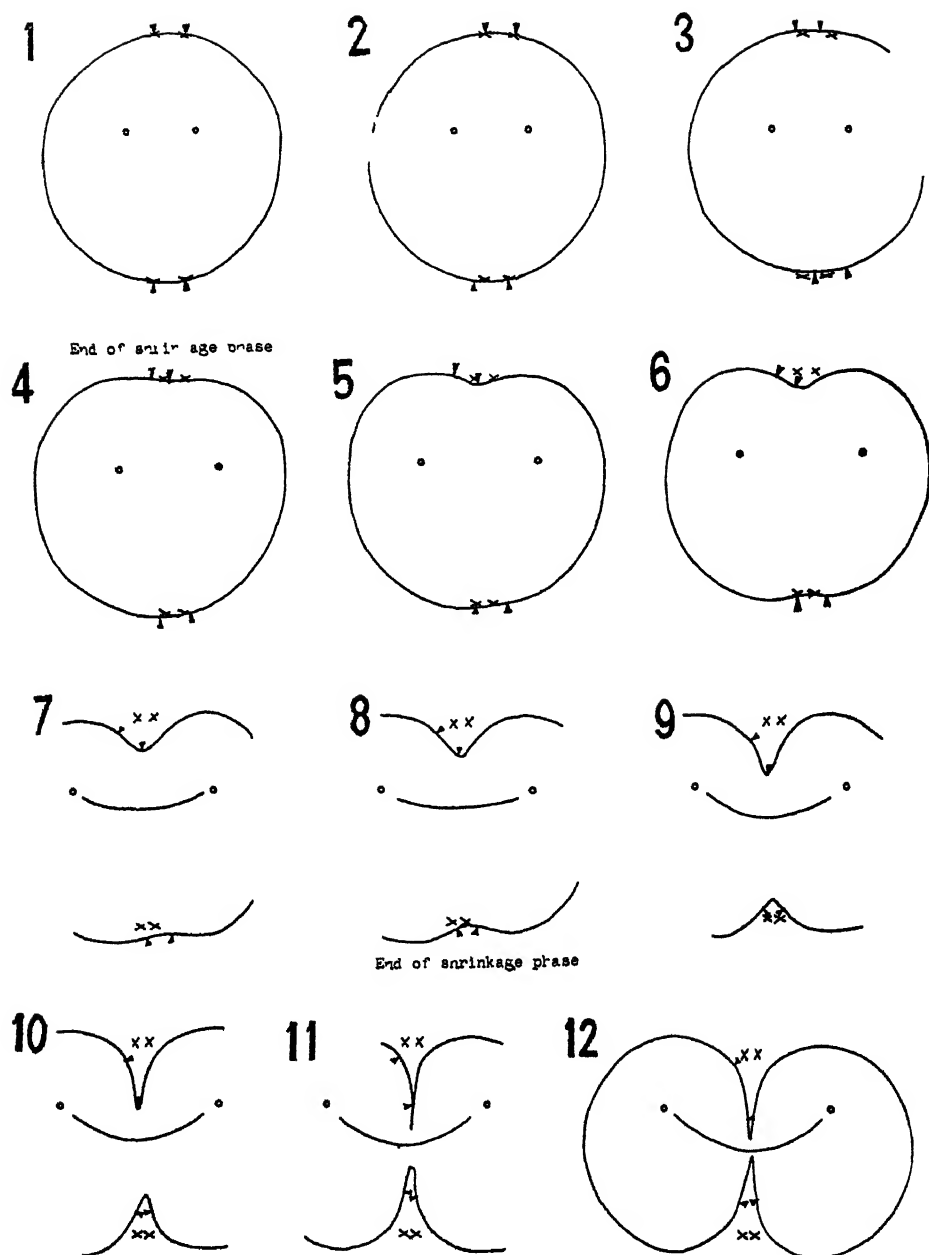


FIGURE 10. Geometrical analysis of an egg with a less excentric spindle. Both the animal and the vegetal poles undergo a shrinkage. The vegetal intersection points are inside the drawings at the seventh and the eighth stages with bent spindles.

TABLE II

Data of the geometrical analysis of an *Asirichlypeus* egg with a less excentric spindle (see Fig. 10). Designations same as Table I.

Stage	1	2	3	4	5	6	7	8	9	10	11	12
Time of recording at 22.5° C.	0"	1'00"	2'15"	2'30"	3'15"	4'00"	5'00"	6'40"	7'35"	8'35"	10'00"	11'35"
Animal furrow	P = 100.0 D = 100.0	P = 90.6 D = 88.2	P = 78.1 D = 88.2	P = 58.5 D = 66.4	P = 85.1 D = 54.6	P = 75.0 D = 56.2	P = 107.8 D = 46.8	— —	— —	— —	— —	— —
Vegetal furrow	P = 100.0 D = 100.0	P = 100.0 D = 97.7	P = 98.4 D = 88.7	P = 93.2 D = 73.1	P = 97.7 D = 62.4	P = 91.7 D = 63.9	P = 83.4 D = 50.3	P = 81.9 D = 50.3	P = 84.2 D = 45.8	P = 121.0 D = 45.1	— —	— —



offer the best single explanation for both the fluctuation phase and the inward shift of the vegetal intersection points.

In the second paper of this series, the authors (Dan, Dan and Yanagita, 1938) handled the data of the kaolin experiments on the eggs of this sand-dollar as if there was no qualitative difference between the behaviors of the two poles of the cell. Even though they are sure that this omission did not affect the conclusion of that paper materially since they were not limiting themselves to the extremely excentric cases, they wish to make this point clear.

Finally, the eggs with less excentric spindles must be examined. In one sense, this test is very important because in eggs with less excentric spindles, their bending can be noticed only in a very late stage of the cleavage process. The data are given in Figure 10 and Table II. As can be seen from the table, the particle-distance on the animal furrow reaches its minimum at the fourth stage. Until this time, the intersection points remain on the contour. The vegetal furrow, in this case, undergoes a shrinkage similar to other examples reported in the previous paper. In this particular case, the vegetal furrow remained in the shrinkage phase for an exceptionally long time, i.e., until the eighth stage. Yet the intersection points stay on the contour through this period, except the seventh and the eighth stages where the vegetal intersection points are inside the contours concurrent with the onset of spindle bending. On closer examination of Table II, however, it will be noticed that the degree of shrinkage is much less for the vegetal furrow (minimum—81.9%) than for the animal furrow (minimum—58.5%). Is this, then, due to the pulling loose of vegetal rays as a result of astral rotation? Here, the stricter test—i.e., the numerical fit between the theoretical and observed degrees of shrinkage—must be resorted to. It was pointed out that these figures coincide only when the positions of the kaolin particles fall exactly at the tips of the crossing rays. This occurs purely by chance. But if numerous records are examined, such cases will inevitably be met with. This is true with the animal pole of the *Astriclypeus* eggs as shown in Table III and Figure 11.

On the contrary, for the vegetal furrow, no case of an exact fit has been found in the entire mass of records. In every case, the degree of the observed shrinkage was far less than that of the theoretical shrinkage, no matter how small a particle-distance was chosen. (When a particle-distance is wider than the actual crossing range, the observed degree of shrinkage becomes less than that of the theoretical shrinkage (consult Figure 2).) The case given in Figure 10 and Table II well exemplifies the situation. This is decisive enough evidence that the asters must

TABLE III

An example of a nearly perfect fit between the observed (*P*) and the theoretical (*D*) degrees of shrinkage at the animal furrow of the *Astriclypeus* egg (see Fig. 11).

Stage	1	2	3	4	5	6
Time of recording	0"	2'05"	3'10"	4'00"	6'25"	6'55"
Observed shrinkage	<i>P</i> = 100.0	<i>P</i> = 93.0	<i>P</i> = 89.5	<i>P</i> = 83.7	<i>P</i> = 73.2	<i>P</i> = 131.3
Theoretical shrinkage	<i>D</i> = 100.0	<i>D</i> = 96.5	<i>D</i> = 88.3	<i>D</i> = 81.3	<i>D</i> = 69.8	<i>D</i> = 58.1

be rotating also in the eggs with less excentric spindles where the early detection of spindle bending is difficult.

In short, the above result indicates that in the early stages of cleavage of the cases of less excentric spindles, the asters are rotating although spindle bending is not detectable. This may mean that while, as was pointed out above, the connection between the spindle tip and the astral center is surprisingly firm, it is not absolutely rigid and a certain degree of astral rotation may be induced by the pressure of the furrow on the sheath rays before the spindle is noticeably affected.

#### SPINDLE BENDING IN OTHER FORMS

The bending of the spindle in *Astriclypeus* has been discussed and is shown diagrammatically in Figure 7. Other observations of spindle bending are not scanty in the literature. Yatsu's last drawing clearly illustrates it for *Cerebratulus*. Among other egg cells, Conklin (1902) shows many drawings of spindle bending in

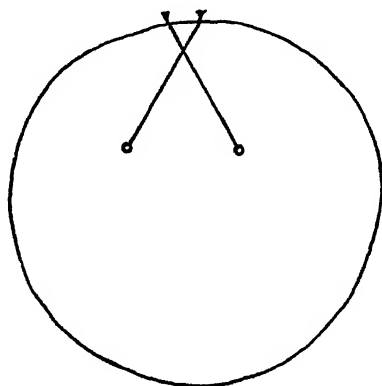


FIGURE 11. Sketch of the egg, the data of which are given in Table III, showing the range of crossing of the rays at the animal pole.

*Crepidula* eggs (Plate III, Figs. 60, 61; Plate V, Figs. 80, 81), Kuhn (1913) reported it in the summer eggs of *Polyphemus* (Fig. 15) and Rhumbler (1901) in the eggs of *Rhabdonema* (?). From both the drawings and the original texts of these reports, it is evident that the spindles lie excentrically in all cases.

Examples of spindle bending are also found among male sex cells. Wilson takes up this subject in his book (1925, p. 140) in connection with the division of the spermatogonia of the grasshopper, *Rhomaleum*. Among recent workers, Sato (1940) touches this point in the spermatogonia (Figs. 18, 48) and the spermatocytes (Figs. 20, 50) of the scorpion, *Buthus martensii*, and he discusses it as a possible cause of the rosette arrangement of these cells within their cysts. He kindly informed the authors in a personal communication that, in these cases, the position of the resting nucleus and subsequently of the spindle is excentric. He also asserted that the bending of the spindle is not due to a lack of space within the cyst. From the above references, the authors believe there is enough evidence to support the proposition that spindles bend whenever they occur excentrically in cell bodies. As a matter of fact, once aware of this situation, careful observation can frequently

catch slight bending of the spindle even in the so-called "regularly dividing" sea-urchin eggs, in batches in which the spindle lies more or less off the geometrical center of the cell (*see* Fry, 1937; Figs. 1-5). Another fact of special importance is that some of the investigations cited above include figures of spindle bending which occur well before a cleavage furrow is formed, thus definitely eliminating the possibility that the spindle is passively bent inward by the advancing cleavage furrow.

#### ARTIFICIAL INDUCTION OF SPINDLE BENDING

The foregoing analysis furnishes evidence that the cause of spindle bending is an unbalanced condition of the mechanical strains between the animal and vegetal sides of the spindle. If such is the case, by experimentally causing a similar unbalanced condition of force, it should be possible to induce spindle bending in a material in which it is not seen normally. This is achieved by the use of ether. The eggs of any kind of echinoid which normally divide in a symmetrical fashion can be used. The eggs are fertilized and a short time before the first cleavage is due, the surrounding sea-water is changed to an ether-sea-water mixture (0.6% by volume).

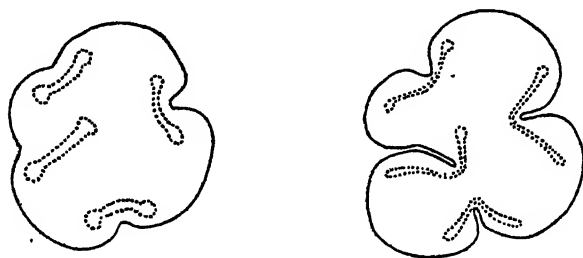


FIGURE 13. Reproduction of Yatsu's Figures 22 and 23 of a *Cerebratulus* egg, whose previous mitoses were inhibited by compression, about to cleave into four binucleate cells. The bending of the spindle is clearly shown.

In ether, even though the nuclear division takes place, the cytoplasmic division is suppressed and binucleated cells are obtained (Wilson, 1901). If these eggs are later washed free from ether, at the second cleavage, two spindles are formed side by side. Under this circumstance, the cleavage furrow appears in a plane at right angles to these spindles and two binucleate cells result. As the furrow constricts the cell, each of the two parallel spindles will obviously be cut by the furrow only on its peripheral side. In the living condition, during the cleavage, two obtusely V-shaped spindles can be seen which, at the final stage, are held in contact at their apices, giving the form of an X. Sections of ether-treated *Strongylocentrotus* eggs at the second division are shown in Figure 12.

In the literature, Boveri (1910) sketched a bent spindle in an *Ascaris* egg, the cleavage furrow of which was suppressed on one side by centrifugation. Yatsu (1908), in his experiments on *Cerebratulus* eggs with a compressorium, shows clear figures of spindle bending in an egg which is dividing simultaneously into four binucleate cells, even though his interest in that paper is on other points. Two of his figures are shown in Figure 13. This example suggests that whenever a cell divides simultaneously into many cells, spindle bending must be involved.

## DISCUSSION

In the foregoing sections, the authors have tried to analyse the cause of the bending of excentric spindles, and they have attributed it to the unbalanced mechanical resistance on the two sides of an elongating spindle resulting from its ex-

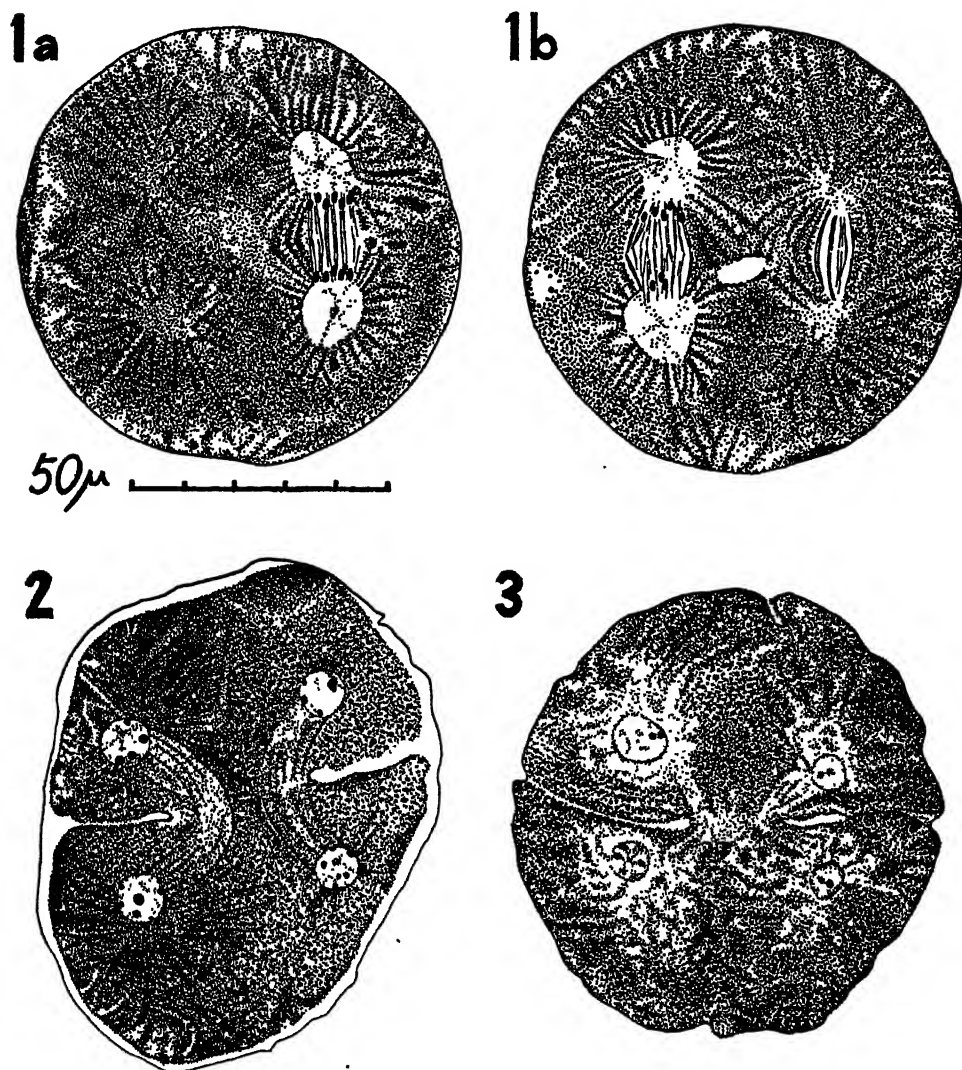


FIGURE 12. Sections of an egg of *Strongylocentrotus pulcherrimus* in the second division, the first cleavage of which was suppressed by ether. Fixed in chrome-formol mixture, 1a and 1b are two successive sections of an egg showing two spindles and four asters (the slit in the middle of 1b is an artefact). Two is an egg cleaving into two binucleate cells with the bending of the spindles. Three is another egg in a slightly later stage with sharply bent spindles, the spindles being much more vague in this case.

centricity. As far as the authors are aware, Gray is the only investigator who has tried to derive an explanation of this mode of division from a general theory on the division mechanism of regularly dividing cells. In discussing "astral cleavage" (Gray, 1931, pp. 201-202), he writes: "If the line joining the center of the two asters does not pass through a diameter of the egg by the time the astral rays reach one side of the egg, then the cleavage furrow develops first on that side, and only later (as the rays reach the opposite side of the equator) does the furrow develop on the other side. . . . That the irregularity of form and position of the asters is the cause and not the result of irregular cleavage is suggested by the fact that similarly irregular cleavages can be induced to occur in *Echinus* eggs by experimental means."

Although the authors readily support the latter part of his statement, they cannot help thinking that the mechanism he offers as the explanation for the formation of one-sided furrows is quite unsatisfactory. In the first place, his theory requires the existence of a stage in which the rays are reaching the nearer periphery but not the more distant side. This is quite contrary to the observed facts. It is obvious that the furrow formation starts with the elongation of the cell body only after the astral rays have filled up the cell interior. In the second place, even allowing the first requirement of Gray's theory, the way in which the furrow appeared on the nearer side by his mechanism would not be the formation of a furrow in the real sense of the words but would rather be the bulging out of the two sides of the furrow. Consequently, at the completion of such cleavage, the egg should have made headway toward the animal side, causing the spindle to take a central position. This is utterly incompatible with the observed facts.

A striking similarity between *Astriclypeus* and *Cynthia* eggs concerning spindle bending and the vortex configuration of the astral rays has been mentioned. However, the real situation seems to be much more complicated. Conklin (1905) states that the cleavage furrow of the 1st division starts from the vegetal pole near which the clear protoplasm and the mitotic apparatus are lying excentrically (see Conklin, 1905; Pl. II, Fig. 20). So far nothing contradicts the authors' expectations. But he further says that when the spindle begins to bend, toward the end of this cleavage, it becomes convex toward the vegetal pole from whence the furrow first appeared (Pl. VII, Fig. 100). He moreover asserts that this is also true for the first cleavage of *Ciona intestinalis* (Pl. XI, Fig. 198). The direction of this bending is quite opposite to the one which can be expected from our theory.

On the other hand, for the second and third cleavages, the direction of the spindle bending is exactly what we would anticipate. He writes: "In the second cleavage the constriction of the cell begins at the periphery or free surface and proceeds inward through the cell body (figs. 104, 105). . . . and the middle of each spindle is bent in toward the center of the egg (fig. 105)" (p. 44). On page 46, he has another sentence to the same effect in connection with the first cleavage and with Figures 106, 107. A reexamination of ascidian egg cleavage is urgently needed.

From what has been discussed concerning the cause of spindle bending, it might be concluded that spindle bending is impossible in ordinary plant cells in which neither astral rays nor cleavage furrows are found. In spite of this, there are cases in which spindle bending has been reported among plant cells. Professors Sinotô and Wada of the Botany Institute kindly informed the authors of two such cases. One is in the pollen mother cells of a haploid *Triticum* reported by Gaines and Aase (1926) and the other is in the pollen mother cells of hybrid offspring of

*Triticum* and *Aegilops* studied by Kihara and Lilienfeld (1932). The cause of such bending may be quite different from that among animal cells and it must probably be sought for in the intrinsic make-up of the spindle itself.

The authors are happy to express their gratitude to Dr. Laura Hunter Colwin for her help in preparing this and the following manuscript, and to Dr. D. P. Costello and Dr. Daniel Mazia for their criticism and advice.

#### SUMMARY

1. Study was made of the cleavage of cells with excentric mitotic figures.
2. In the division of such cells, (a) the cleavage furrow appears from the side of the cells nearest to the mitotic figure, (b) the asters rotate, causing the animal hemispheres to converge, and (c) the spindle bends, with the convex side directed toward the vegetal pole.
3. This situation can be explained by thinking that the mechanical resistance for the elongating spindle is different in magnitude on its two sides as a result of the excentricity of the mitotic figure.
4. The above situations can be artificially induced in materials which never show them normally, by producing a similar unbalanced resistance on the two sides of the spindle.
5. Gray's theory concerning the mechanism of the formation of one-sided cleavage furrows is discussed.

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## BEHAVIOR OF THE CELL SURFACE DURING CLEAVAGE. VIII. ON THE CLEAVAGE OF MEDUSAN EGGS \*

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Coelenterate eggs cleave characteristically with a one-sided advance of the furrow on the animal side, in contrast to utter quiescence on the vegetal side.<sup>1</sup> Around the beginning of the present century various theories were proposed to account for the mechanism of such division but they were highly hypothetical in nature, and failed to acquire much popularity. The present study, although admittedly preliminary in nature, is an attempt to clarify some aspects of the division of medusan eggs.

In previous papers the authors have postulated that the primary cause of cleavage is spindle elongation, both in regularly cleaving eggs such as those of the sea-urchin (Dan, 1943b) and also in cells with excentric nuclei (Dan and Dan, 1947). But in the asymmetrical furrow formation of eggs of the latter type, for example in the heart-shaped cleavage of *Astriclypeus manni*, there are secondary peculiarities not found in regular cleavage. The first is rotation of the asters about their centers, so that their respective animal hemispheres converge. This results from asymmetrical sheath ray formation, and can be detected by noting the change in direction of bending of the rays in successive stages of cleavage (Dan and Dan, 1947). The second peculiarity is the bending of the spindle, so that it becomes convex toward the vegetal pole. This is a direct result of the astral turning. Of course, such bending is possible only because the spindle itself is somewhat rigid, and the connections between the spindle tips and astral centers are firm.

Now, among different batches of *Astriclypeus* eggs, the excentricity of the spindle varies and the degree of one-sidedness of the furrow varies correspondingly. An orderly arrangement of these eggs, from less to more excentric spindles, will form a series continuous with the mode of cleavage of medusan eggs (Fig. 1). This might mean that medusan cleavage simply represents an extreme case of heart-shaped cleavage. If so, astral rotation and spindle bending should also be found in medusan eggs. The following investigation will test these propositions. Moreover, a few points specific to the present material will be presented.

\* A part of this work was aided by a fund offered by the National Research Council of Japan, for which the authors' deep thanks are due.

<sup>1</sup> Opinions differ as to which side of the medusan egg should be considered the animal pole. Ordinarily, the side at which the polar body is extruded and the cleavage furrow begins is called the animal pole. Schleip (1929) and Korschelt (1936), however, have adopted the opposite terminology. This is because the egg axis and the larval axis run in opposite directions among the Ctenophora. In order to avoid confusion, the terms "polar body pole" and "micromere pole" are sometimes used. In this paper, the term "animal pole" denotes the polar body pole and the term "vegetal pole" refers to the micromere pole, thus preserving a unity of expression throughout this series of papers.



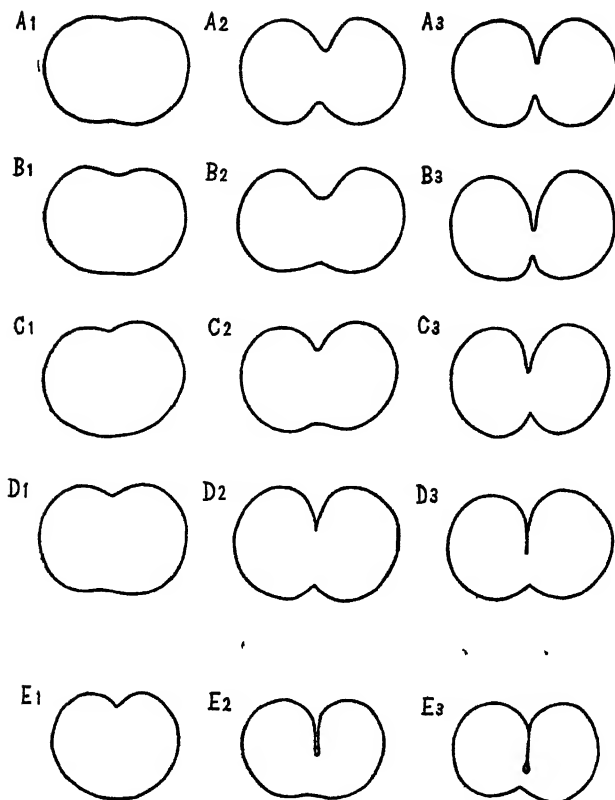
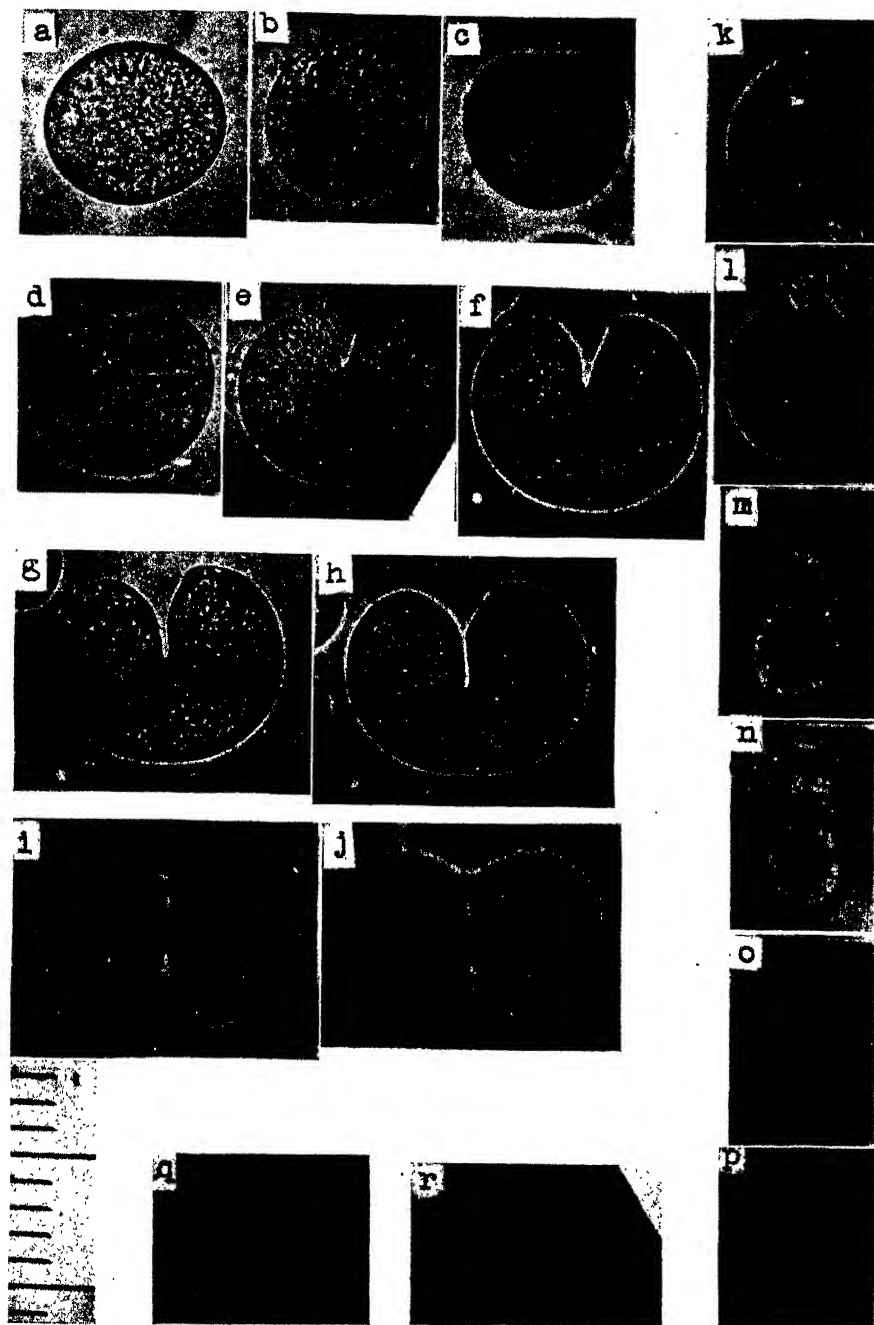


FIGURE 1. Comparison of the cleavage modi of sand-dollar eggs (*Astriclypeus manni*) and medusan eggs (*Spirocodon saltatrix*). A-D, cleavage of sand-dollar eggs arranged in order from less excentricity to more excentricity of the mitotic figure. E, cleavage of the medusan egg. Note that these make a continuous series.

The eggs of *Spirocodon saltatrix*, one of the *Tubularioanthomedusae*, are used throughout this work. This medusa is quite abundant in the bays around the Misaki Station during the winter. The animals spawn on being put in the dark.

PLATE I. (a) Short time after fertilization. The egg pronucleus is vaguely visible at the extreme left side of the cell, which is the animal pole. (b) Conjugating pronuclei and the resulting mitotic figure are invisible by the usual microscopic observation. (c) and (d) Beginning of furrow formation. (e) Slightly more advanced stage. (f)-(j) Successive stages of cleavage of the same egg. (f) The spinning is not yet evident. (By this time, the fine protoplasmic processes must be coming out from the furrow surface; these are not visible unless observed under oil immersion.) (g) The amalgamation of the protoplasmic processes is partly completed. (h) (i) (j) The amalgamation products spread between the blastomeres in a sheet, fastening the cells together. (k)-(p) Views along the spindle axis. (k) (l) Initial stages of cleavage which roughly correspond to stage (e) of the side view. (m) More advanced stage. Note that the still uncleaved part between the blastomeres can be vaguely seen as a bright circle. (n) (o) (p) Successive stages of cleavage of a single egg. Notice that as the connecting bridge narrows down, the blastomeres round up. (q) View from above (animal pole). (r) View from below (vegetal pole). The smallest division of the scale is 10 $\mu$ .

PLATE I



(Uchida, 1927). The reaction is particularly clear-cut if the animals have previously been exposed to electric light. Around  $10^{\circ}\text{C.}$ , the first cleavage takes place from  $1\frac{1}{2}$  to 2 hours after putting them in the dark, depending upon the latent period for spawning.

These eggs have absolutely no enveloping membrane in either the unfertilized or fertilized condition, as far as can be judged by microscopical observation. The egg pronucleus is clearly visible in the unfertilized condition and vaguely so during a short time after fertilization, taking an extremely excentric position within the egg (Plate Ia). But by the time syngamy approaches, it is lost from view (Plate Ib). When mitosis begins, the asters are discernible, although the rays are very faint and require oil immersion to be definitely distinguished (Plate II). For this reason, sectioning is necessary for detailed observation of the internal structures. When unfertilized eggs of *Spirocodon* are fixed in a chrome-formol mixture, the internal cytoplasm remains fairly homogeneous throughout the cell (Fig. 2; 1). But if eggs are fixed by the same mixture after fertilization, the peripheral cytoplasm assumes a coarsely vacuolar aspect (Fig. 2; 2, 3, 4, 5). On the other hand, if fixed by osmium, the cytoplasm of fertilized eggs is homogeneous (Fig. 3). From the above facts, it can be assumed that the peripheral vacuoles of the chrome-formol-fixed eggs are artifacts. If fertilized eggs are over-stained by neutral red or by Nile blue sulphate, many large heavily stained vacuoles appear in the peripheral zone of the cells. Such over-stained cells have already lost the capacity to cleave.

#### NORMAL CLEAVAGE OF THE EGGS OF SPIROCODON

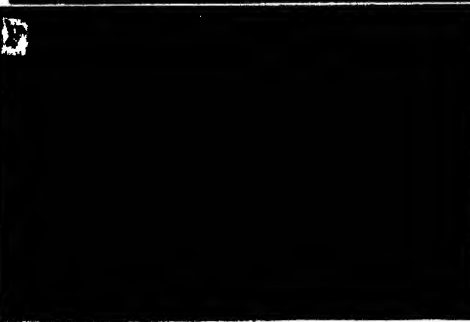
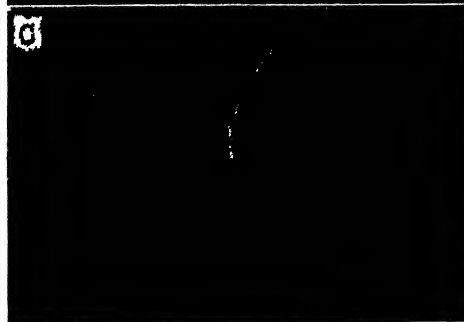
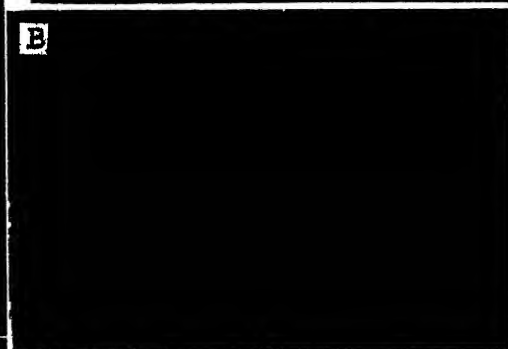
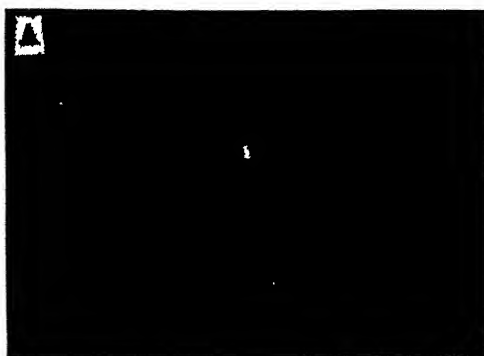
Since the asters of this egg cannot be seen with certainty unless oil immersion is used, it is rather difficult to anticipate the exact time of the onset of cleavage. Most observations, therefore, were started after a slight indication of furrow formation had been noted. This drawback was somewhat offset by the slowness of the advance of the furrow.

When the furrow deepens, the egg tends to come to lie on its side, with the egg axis parallel to the substratum. As for the changes in contour in the side views of these cells during cleavage, there is not much to add to what has been reported for other forms (Plate I, *b* to *j*). If viewed from other angles however, an interesting feature appears. Photographs of two views perpendicular to the side view and to each other are shown in Plate I, *k* to *r*. From these, it is clear that cleaving *Spirocodon* eggs form flat plates of squarish shape. This is the reason why they tend to come to lie on their sides. Although sea-urchin eggs elongate in the direc-

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PLATE II. Successive stages of cleavage of a single *Spirocodon* egg observed with oil immersion lens. (A) Slight indication of the vortex figure within the blastomeres. No sign of the spinning activity within the furrow. (B) The vortex configuration is becoming more evident. The spinning activity has not begun yet. (C) The vortex figure is at its climax. The amalgamation of the protoplasmic processes is proceeding (see C'). (C') Enlargement of C showing the spinning activity in the furrow. Note a longish shadow along the furrow space and several very faint lines running across the furrow, nearer the tip of the furrow. The latter are threads formed by the fusion of several protoplasmic processes which cannot be shown in the photograph. The former is the amalgamation product of the latter. This grows by further amalgamation into a sheet. (D) The spinning is complete around the entrance of the furrow. (E) Astral rays (vortex figure) are becoming vaguer. (F) The furrow has almost reached the vegetal side. The smallest division of the scale is  $10\mu$ .

PLATE II



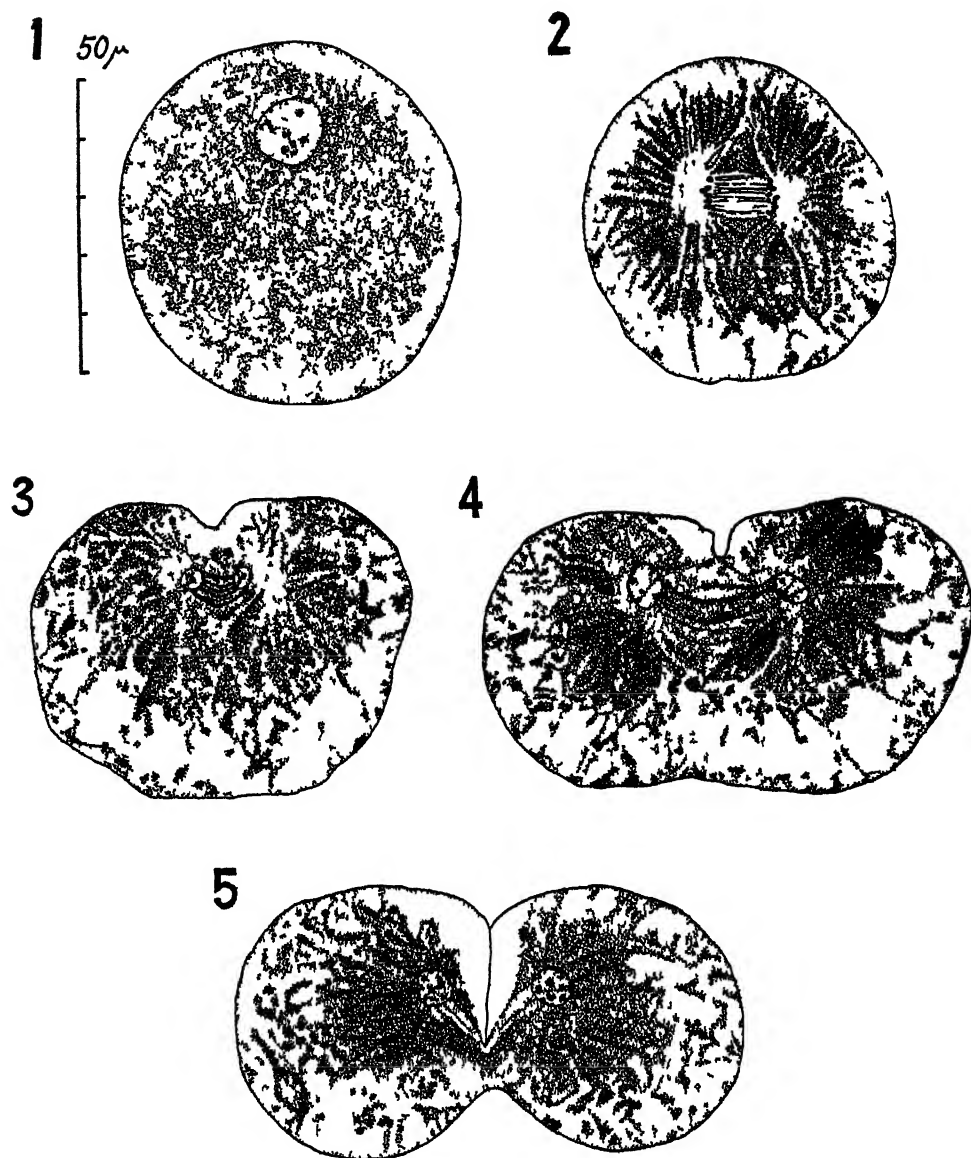


FIGURE 2 *Spirocodon* eggs fixed in chrome-formol mixture 1 unfertilized egg The cytoplasm is fairly homogeneous with only a slight tendency to vacuolization around the periphery 2 telophase with chromosomes and asters Note an intense vacuolization around the periphery The plane of section is quasiequatorial, the peripheral vacuoles are seen encircling the island of dense cytoplasm 3, vertical section Anaphase with asters, a bending spindle and a shallow furrow Notice that the island of dense cytoplasm is definitely shifted toward the animal pole 4, a still later stage 5, a much later stage with a V-shaped spindle Note that the vegetal vacuoles have now come around to the former animal side

tion of the spindle, the shape of cross-sections parallel to the cleavage plane is always circular. Similar cross-sections of medusan eggs are a series of oblongs (Plate I, *m*, *n*, *o*), and only in later stages do the flattened incipient blastomeres gradually round themselves up (Plate I, *o* and *p*). This flattening is an important point to keep in mind for an understanding of the division mechanics of coelenterate eggs.

Several other things are notable in the side view of the egg. For example, in every case observed, a vortex configuration can be seen in each blastomere during most of the cleavage process (Plate II, *B*, *C*, *D*, *E*). The direction of the whorl of this vortex is exactly the same as that exhibited by the astral rays of *Astriclypeus* eggs (Dan and Dan, 1947; Fig. 7), only the degree of bending of the component lines is much more acute in the *Spirocodon* egg.

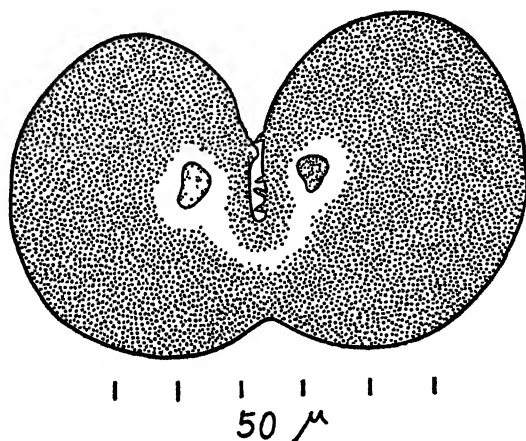


FIGURE 3. *Spirocodon* egg fixed in Flemming's solution showing homogeneous cytoplasm, V-shaped spindle and spinning processes on the furrow sides.

The next fact concerns an utterly new feature of cell activity which finds no similar case among echinoderm eggs. This is the so-called "spinning activity" of the medusan eggs.<sup>2</sup> It has been known for some time that in medusan eggs, after the cleavage furrow advances some distance, fine protoplasmic processes are sent out from the furrow sides to amalgamate into a sheet between the two blastomeres (Yatsu, 1912; Plate II).

As material for observing this spinning activity, *Spirocodon saltatrix* is not particularly favorable, for the processes are so fine that, before amalgamation, they are barely recognizable individually with an oil immersion lens (Plate II, *C'*). But the sheet, once it begins to be formed (Plate II, *D*, *E*), can be seen with the usual high dry objective (Plate I, *g*, *h*). Moreover, the partly amalgamated processes can be fixed as such by osmium (Fig. 3). Unfortunately, because of the poor visibility of *Spirocodon* processes, the details of the change from processes to sheet cannot be

<sup>2</sup> Andrews (1897) and Moore (1930) discuss spinning activity in connection with echinoderm eggs. Their views will be criticized in a future paper.

ascertained. All one can say is that the transformation is rather abrupt and as the sheet is completed, it spreads between the opposed surfaces of the blastomeres like a wetting fluid, sometimes even showing a meniscus at the optical section of its border. As the immediate result, the two blastomeres are pulled closer to each other.

The last point concerns the final positions of the divided nuclei. In either unfertilized or fertilized eggs, as long as a nucleus is visible in the living condition, it takes an extremely excentric position toward the animal pole. After it is lost from view and the mitotic spindle develops, sections show that even though the spindle has come back to a more central position, it is still decidedly pushed to the animal pole (compare Plate Ia and Fig. 2, 3 and 4). Nevertheless, when the two resting nuclei are reconstituted after cleavage their positions are about midway between the animal and vegetal poles, almost in contact with the furrow sides. This situation is quite different from that existing in *Astriclypeus* eggs, where the nuclei retain the same degree of excentricity until the four-cell stage.

Now let us see how the facts described above can be correlated with the general theory of cleavage developed in the previous papers.

#### ROTATION OF THE ASTERS

The direction of the whorl of the vortex configuration in the dividing blastomeres of *Spirocodon* has been shown to be the same as that exhibited by the astral rays of *Astriclypeus* eggs. In the latter case the vortex figure of the astral rays was taken as a direct indication that the asters are turning around their centers (Dan and Dan, 1947). Examination under oil immersion leaves no doubt that the figure in the *Spirocodon* eggs is also primarily due to the bending of the astral rays. Consequently, it is safe to say that the vortices in the two forms are identical in significance. Judging from Yatsu's figure (1912; Plate II, Fig. 19), similar vortices are also evident in the eggs of *Beroë forskalii*.

#### BENDING OF THE SPINDLE

In the previous paper, the primary cause of spindle bending in *Astriclypeus* eggs was attributed to the excentric position of the mitotic figure. It is easy to show that a similar situation obtains in the eggs of *Spirocodon*. In the unfertilized condition, the egg pronucleus is clearly visible in the living cell and it lies immediately beneath the cell membrane at the animal pole (Plate Ia). After fertilization, although sections show that the first mitotic figure comes back toward the interior to some extent, still it is clearly excentric. As cleavage proceeds, sections also reveal that the spindle is bending. This is true both for eggs fixed with chrome formol (Fig. 2) and for those fixed in osmium (Fig. 3).

Now one is able to understand why the *Spirocodon* eggs flatten during segmentation. In regularly dividing eggs where the spindle simply elongates, the force acts in linear fashion and the cell body can only elongate along the spindle axis. But in the cleavage of medusan eggs, in addition to the force which pushes the two asters apart, there is another force trying to make them turn around their excentric centers. This set of forces determines a plane on which the eggs tend to spread and make flat discs. This fact is most clearly brought out by making contour drawings

of the side view of the same egg at various stages and superimposing them. As is shown in Figure 4, the outline of the initial stage can be included within that of the flat stage.<sup>8</sup> When the blastomeres round up again in later stages, the contour becomes smaller once more.

In passing, it must be made clear that the above statement that a set of two forces is at work in the medusan cleavage should not be taken to mean that two different factors are involved in the process. As was discussed in the previous paper (Dan and Dan, 1947), the force causing the rotation of the asters is derived from the elongating force of the spindle as a result of the excentricity of the mitotic figure. Therefore, they are nothing but two fractions of force of a single origin.

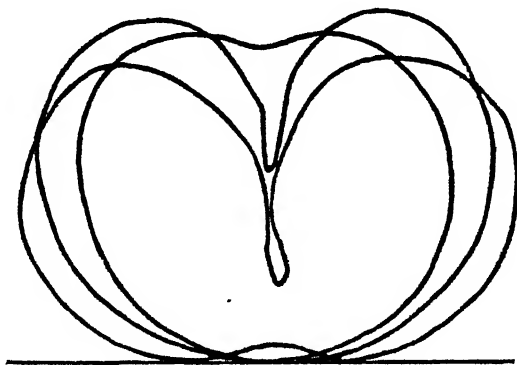


FIGURE 4. Superposition of the contours of the side-view of the same egg in three different stages. The contour of the second stage surpasses that of the first stage both in height and width, indicating that the cell body is spreading flat in this stage. In the third stage, although the width increases, the height decreases, showing that the blastomeres are rounding up.

Returning to the main line of thought, if it be correct that the flattening of the blastomeres and the bending of the spindle are two effects of a single cause, then we should have noticed the same thing in the eggs of *Astriclypeus*. Re-examination of that material shows it to be actually present, as is illustrated in Figure 5. As a matter of fact the bulging out of the vegetal contour was often noticed during that study and its probable cause was attributed partly to spindle bending, partly to astral rotation. The situation would have been grasped more readily if it had been considered in terms of flattening.

#### SPINNING ACTIVITY AND ITS SIGNIFICANCE WITH RESECT TO CLEAVAGE

Once it is established that astral rotation is one of the important factors in medusan cleavage, one is led to suspect that spinning activity may be closely related as a part of the division mechanism, for when the two asters are simultaneously moving away from each other and rotating around their centers, a tying together of

<sup>8</sup> Yatsu's paper includes a somewhat similar figure of the superposition of outlines of successive stages in the cleavage of the eggs of *Beroë*. In connection with this figure, Yatsu is pointing out the absence of lateral elongation in *Beroë* eggs. But it should not be overlooked that the stages he is dealing with are those of the latter half of the division process, and the contour of the early period is not included.



the animal side of the blastomeres would greatly increase the efficacious force available for the tearing open of the vegetal furrow.

The most direct and decisive way to test this possibility is to interfere with the spinning activity and see whether or not the eggs can divide. A method of mechanical intervention was adopted. It consisted in moving a micro-dissection needle back and forth along the furrow as the latter was being formed. It was thought that this method would prevent the amalgamation of the protoplasmic processes. Special care was taken not to injure the sides of the furrow by the needle during the operation. The result was clear-cut. The eggs divided completely. But on completing cleavage, the blastomeres fell apart. Later it was found that a simpler way to achieve this aim is to make the eggs divide under compression in a hanging drop with an extremely small quantity of sea water. The tracings of photographs shown in Figure 6 illustrate an egg so treated. The reason for the failure of the spinning under compression is probably that, as the egg is flattened so much, the

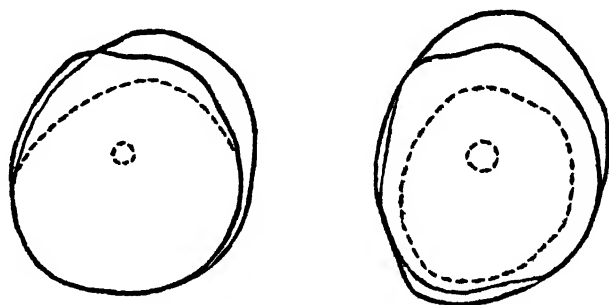


FIGURE 5. Two camera lucida drawings of dividing *Astriclypeus* eggs as seen along the spindle axis. In the left hand one, the furrow is formed only at the animal pole while in the right hand one, the furrow has gone around the cell. The large dotted circles are optical sections of the still uncleaved parts and the smaller dotted circles are cross-sections of the spindle.

number of the protoplasmic processes which are formed just at the flattened edges of the furrow sides is greatly reduced so that the chance of their meeting and consequently of their amalgamation becomes very slight.

At any rate, it can now be said definitely that the sole object of the spinning activity is to tie the blastomeres together and it can be segregated from the process of cell division proper.

#### PERFORATION EXPERIMENTS

In order to get some insight into the more dynamic phase of medusan cleavage, perforation experiments were made. The method is to take an egg compressed in a hanging drop and punch it through by a micropipette (Dan, 1943a).

The results obtained in sea-urchins by this method can be summarized briefly. When a perforation is made through the cytoplasm on the median plane bisecting the karyokinetic figure, the inner border of the perforation is pulled in and becomes a cleavage furrow. If a hole is shifted slightly to the side of the median plane, but still within the crossing range of the median rays (sub-median region), a furrow

is formed at the normal position, but simultaneously the perforation is drawn toward the median plane and the two are ultimately united, so that in the end a nearly-normal two-cell stage with a forked furrow results. If the hole is further pushed toward the polar region, it can no longer take part in the furrow formation.

When *Spirocodon* eggs are compressed for punching, because of the excentric position of the mitotic apparatus it is desirable to have the egg axis coincident with the plane of flattening. But unfortunately, even after compressing, the mitotic figure cannot be located definitely because of its poor visibility. Theoretically, the apparent position of the mitotic apparatus under compression can be the most excentric when the egg axis is lying parallel to the plane of flattening and it becomes

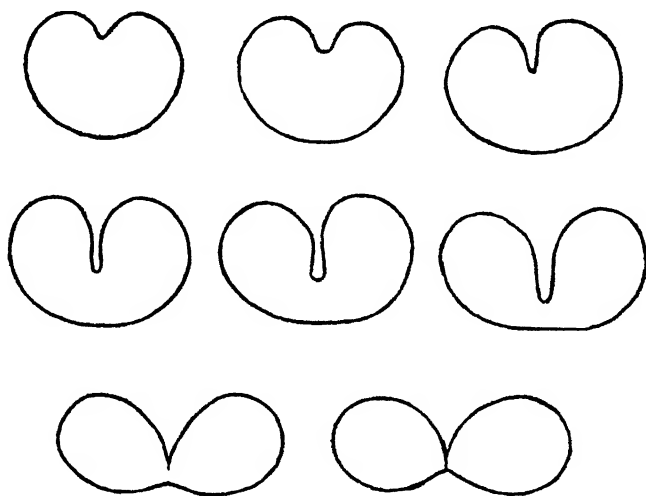


FIGURE 6. Tracings of a series of photographs of a single egg dividing under compression. Spinning has failed and the blastomeres are falling apart. In later stages which are omitted here, they are completely separated. Note, however, that until the cleavage is nearly completed (down to stage 5), the contour change of the blastomere is quite normal, indicating that these shapes are brought about by purely internal causes.

central when the egg axis is perpendicular to the plane. Another source of ambiguity lies in the fact that when a micropipette punches through the cell in the vicinity of the mitotic apparatus, there is no way of telling to which side of the pipette the mitotic figure will slip away. The final position of the mitotic figure after such treatment in each particular case can be judged to some extent by observing the way in which the furrow appears, but sometimes the authors had to guess it from the later behavior of the perforation.

The series of sketches shown in Figure 7 represents the behavior of a perforation on the median plane of the animal side. Just like the corresponding case in sea-urchin eggs, the inner border of the perforation is drawn in, becoming a cleavage furrow, while the median periphery of the cell which would become a furrow under normal conditions remains unchanged. The rest of the division process proceeds in the usual manner. At the fourth stage of the figure, a few partly amalgamated processes

are seen and, at the sixth stage, the amalgamation is complete. The division is achieved at the eighth stage. Soon after this, the mitotic figures for the second cleavage are formed and the egg cleaves into three blastomeres. This is instructive in confirming the observation that the first cleavage has failed to divide the cell completely.

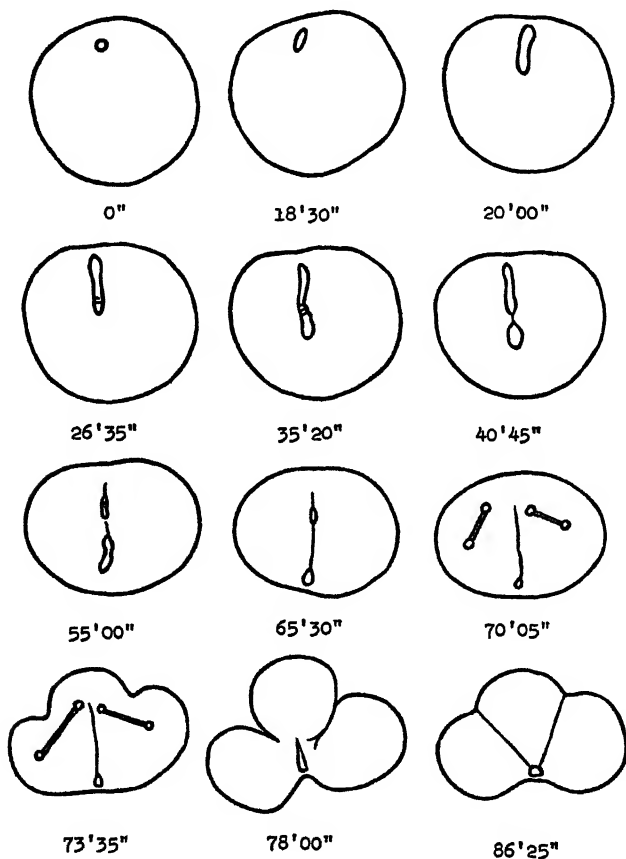


FIGURE 7. Form change of a perforation made on the animal side of the spindle. Note that it makes a cleavage furrow which takes the place of the cell periphery. From the 4th stage on, spinning activity can be seen which succeeds in binding the cells at the 6th stage. At the second cleavage, the larva reaches a 3-cell stage, the middle cell being binucleate and the other two, mononucleate. Temperature,  $14.2^{\circ}\text{C}$ .

The case shown in the next figure (Figure 8) is another example of the perforation of the animal side. In this case, although the result is identical with that of the previous example, as far as the behavior of the perforation is concerned, it is significant in showing a case of the failure of spinning. In this case, since the animal sides of the blastomeres are connected by a protoplasmic bridge, the failure in spinning causes a widening of the tip region of the furrow. As a result, a thread

of cytoplasm running between the blastomeres at the vegetal pole is clearly visible. This string of cytoplasm must be homologous to the connecting stalk of sea-urchin eggs and hence it must contain the spindle, or, more correctly speaking, the spindle remnant, within it. In the present case, however, the apparent elongation of the

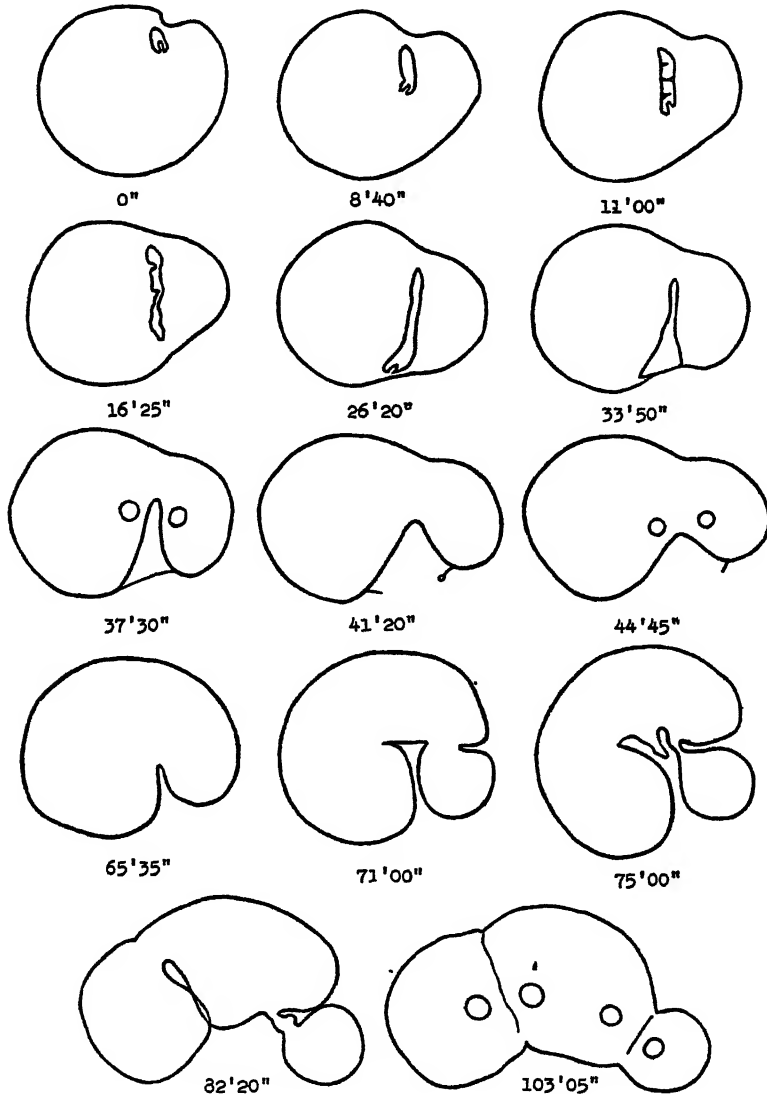


FIGURE 8. Another example of a perforation in the egg at the animal side of the spindle. At the 3rd stage threads are formed which, however, fail to bind the blastomeres. At the 6th stage, the division process is complete. The protoplasmic string left at the vegetal pole is the "stalk" and contains the spindle. At the 8th stage, the stalk is broken. At the 10th stage, the second cleavage sets in, resulting in a 3-cell stage. Temperature, 10.5° C.

spindle remnant cannot be considered to be an autonomous one but is likely a passive one due to the rounding up of the blastomeres. In the second cleavage, the egg divided into three cells, as in the previous case.

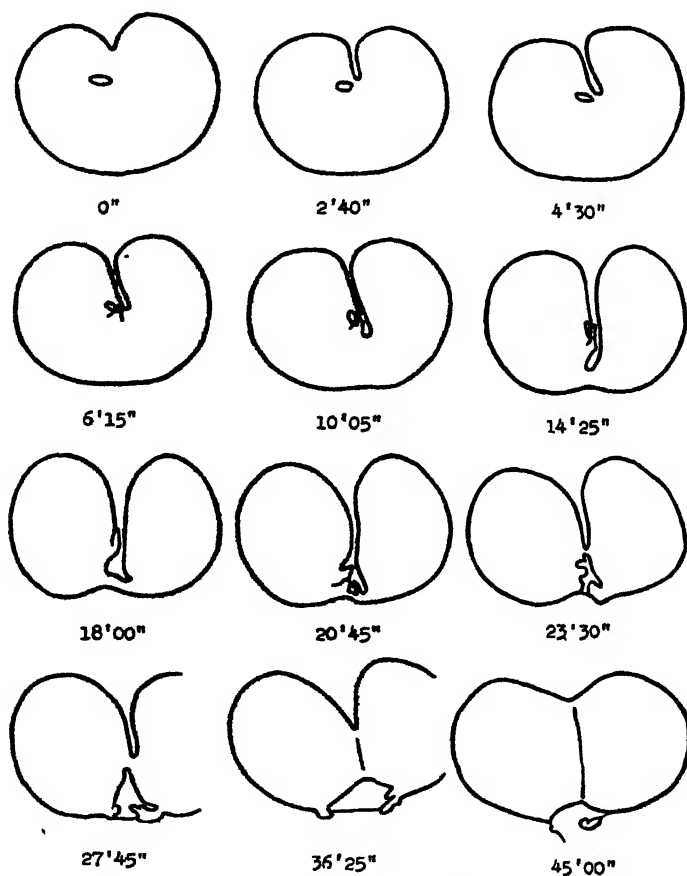


FIGURE 9. The behavior of a sub-median hole on the animal side. Immediately after the operation, the hole was round in shape, but is stretched toward the median plane by the time the 1st drawing was made. At the 4th stage, a protoplasmic protuberance appears in front of the holes. At the 6th stage, the hole is practically fused with the main furrow. At the 7th stage, the hole is stretched, practically becoming a slit so that its fusion with the main furrow cannot be ascertained. But its fusion is evident at the 8th stage through the appearance of a dent on the side of the furrow just above the protuberance. This corresponds to the Y-shaped furrow of *Strongylocentrotus* eggs. At the 9th stage, spinning is achieved at a little higher level than the dent. The spindle remnant is clearly seen at the following 2 stages and is finally broken at the 12th stage as the interkinetic condition is reached. Temperature, 13.3° C.

A typical sub-median case is given in Figure 9. In spite of some irregularities in the contour of the furrow wall, the fusion of the hole with the furrow is evident. The only superficial difference in the sub-median behaviors of the medusan and sea-urchin eggs is probably due to the difference in consistency of the cortex of the two forms.

If a perforation is pushed a little farther from the median plane to the sub-polar region, the hole is only stretched and does not fuse with the main furrow (Fig. 10). If a hole is made in the polar region, it remains quiescent all through the cleavage process (*see* Fig. 14).

In short, as far as the behavior of the perforations on the animal side is concerned, the results are just the same as those in sea-urchin eggs. But once we go to the vegetal side, the reaction changes greatly.

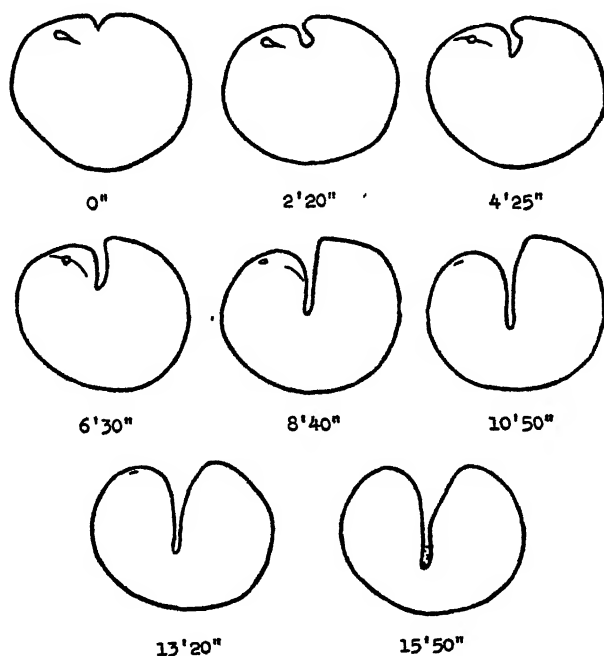


FIGURE 10. A sub-polar hole on the animal side. The hole is pulled toward the median plane. At the 3rd and 4th stages, surface grooves are formed along the direction of strain on both sides of the hole. The hole becomes smaller and slit-like at the 6th and 7th stages and finally disappears at the 8th stage.

The behavior of a median hole on the vegetal side of the spindle is shown in Figure 11. In this case, although the hole is deformed to some extent when the tip of the furrow approaches, there is no sign of the active participation of the vegetal hole in the furrow formation. The situation will more appropriately be described by saying that the furrow advances until it meets a vegetal hole where it simply stops. Therefore, the string of cytoplasm which is found on the animal side in this case must be the spindle remnant.

Here the question arises, whether the behavior of the vegetal hole approaches that of an animal hole if its position is shifted toward the upper pole. Such a case is given in Figure 12. The result is the same as in the preceding case. At the eighth stage of this figure, two nuclei have begun to be reconstructed, but the part of the spindle remnant within the cytoplasm can be recognized as bright streaks

which are continuous with the string of cytoplasm outside the cell. The shallow cleavage furrow thus formed is soon abolished completely and a binucleated cell results. At the second cleavage, the cell showed two mitotic figures. In this particular case, the cleavage attempt is unsuccessful in one blastomere and a 3-cell stage is reached at the end.

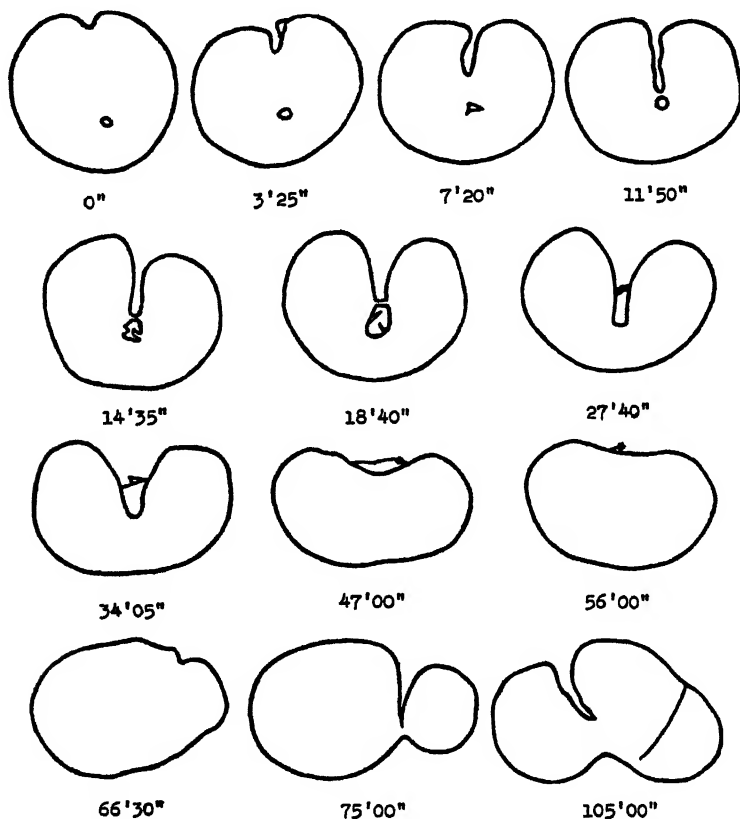


FIGURE 11. A median perforation on the vegetal side. At the 4th stage, the furrow has advanced so that only the stalk containing the spindle remains between it and the hole. At the 8th stage, the stalk is getting soft. The cell is rounding up at the 9th and 10th stages. The cell divides into three at the 13th stage. Temperature, 12.1° C.

Equally striking is the fact that as soon as a vegetal hole is shifted in position from the median plane, it no longer shows any trace of form change and often vanishes leaving only a scar on the cell surface (Fig. 13). In this egg, the hole is obliterated at the third stage, after which the surface scar left by the hole is represented by a dotted line. The instability of the vegetal hole may indicate the fluid nature of this region because perforations in sea-urchin eggs also become unstable when put in a calcium-free medium.

Combination of two holes on the vegetal side of the spindle does not introduce any new type of behavior. In the case shown in Figure 14, one hole is median while

the other hole is polar. The furrow comes down as far as the former and stops there, while the polar hole remains indifferent all through the process. In the case shown in Figure 15, the two holes come pretty close to the furrow, yet they slide along beside it and disappear later.

Summarizing the above results, it can be said that on the animal side of the mitotic figure of the medusan egg, the condition is practically the same as that pre-

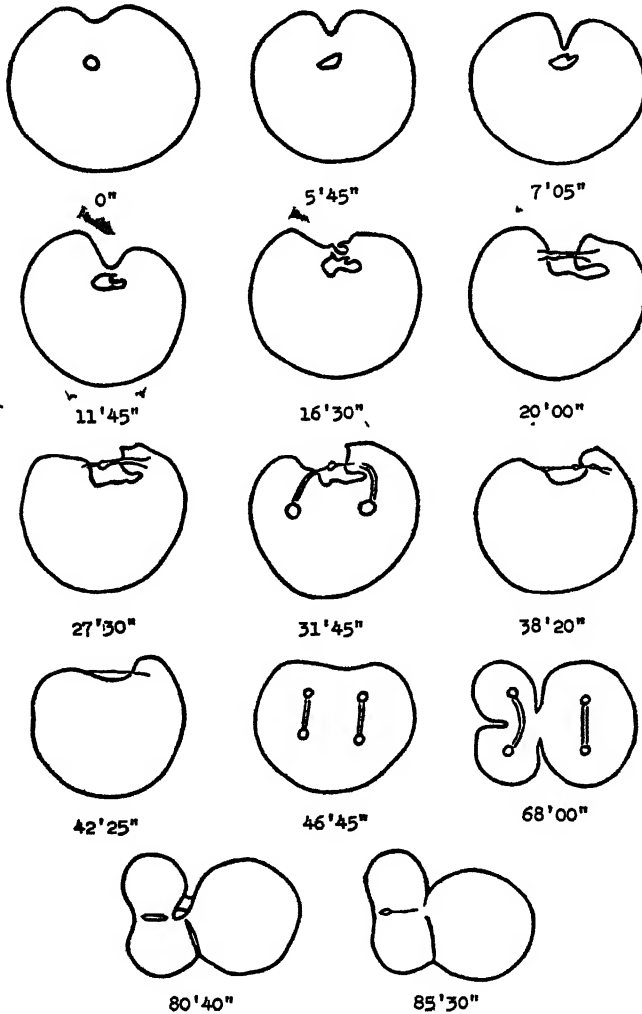


FIGURE 12. A median hole made just below the spindle. The behavior is essentially the same as in the case shown in Figure 11. The 8th stage shows that the string of cytoplasm really contains the spindle. At the 11th stage, two mitotic figures appear. In the right blastomere, the cleavage somehow fails and a 3-cell stage results. The last drawing is significant in showing that the spinning is possible not only between daughter cells but also between adjacent cells which have no common mitotic figure. Temperature, 14.2° C.



vailing in sea-urchin eggs. But the vegetal side is so inert and passive that the situation seems to be utterly different. The impression is unavoidable that the vegetal region is quite fluid. However, if the vegetal region of the medusan eggs is really fluid, is it possible to anticipate a successful cleavage from the proposed theory?

#### THE DIVISION MECHANISM OF MEDUSAN EGGS

As an introduction to this discussion, let us enumerate important features of medusan eggs which are not met with in sea-urchin eggs, and which, consequently,

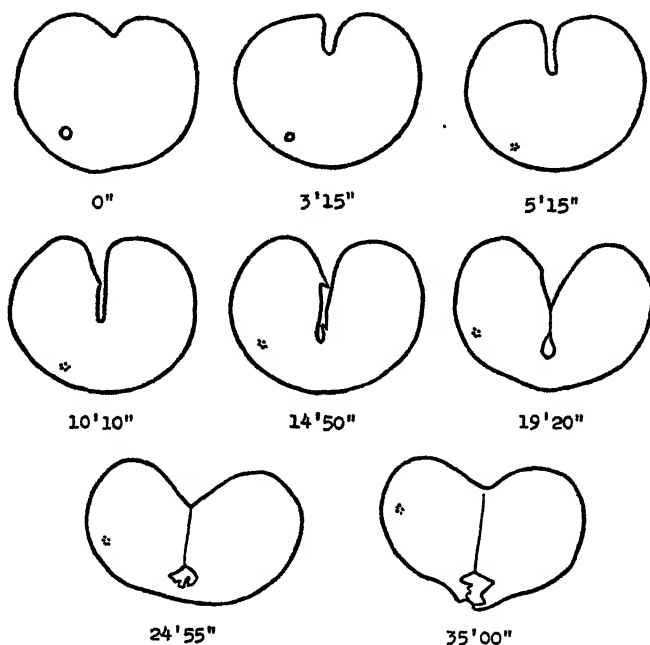


FIGURE 13. A perforation in the sub-median region of the vegetal side of the spindle. At the 3rd stage, the hole vanishes and a surface scar remains. At the 5th stage, the spinning activity is taking place, and at the 6th stage, the two blastomeres are bound together. At the 8th stage, cleavage is complete. Note that the scar is pushed far to the side, in the polar region. Temperature, 12.1° C.

offer basic materials for the present theoretical consideration. (1) Flattening of the cell and later rounding up of the incipient blastomeres. Astral rotation has been offered as a tentative explanation of this fact. (2) Coming around of the vegetal vacuoles to the animal side in fixed eggs and the shifting in position of the surface marks left by vegetal perforations to the apparent polar region after cleavage (*see* texts of Figs. 13, 15). These two facts seem to have a close bearing on the rotation of the asters. (3) Fluid nature of the vegetal region. The instability of the vegetal perforations and the failure of the vegetal rays to exert stress on the perforations are two basic facts pointing to this conclusion. (4) Movement of the nucleus from the animal side toward the vegetal side during cleavage.

With the above four points in mind, the authors have arrived at the following conclusion. In the beginning of cleavage, the spindle begins to elongate as in the case of sea-urchin eggs. Since the astral rays of the animal region of medusan eggs are fully effective, this causes first shrinkage and later furrow formation there just as in sea-urchin eggs. As the furrow gets deeper, the median crossing rays are pushed against the spindle (sheath rays) which causes the asters to rotate. This, in turn, makes the cell spread on a plane determined by these forces of elongation and rotation. When the spindle definitely takes a U-shape, the astral centers must be twisted around through nearly  $90^\circ$  from their former directions.

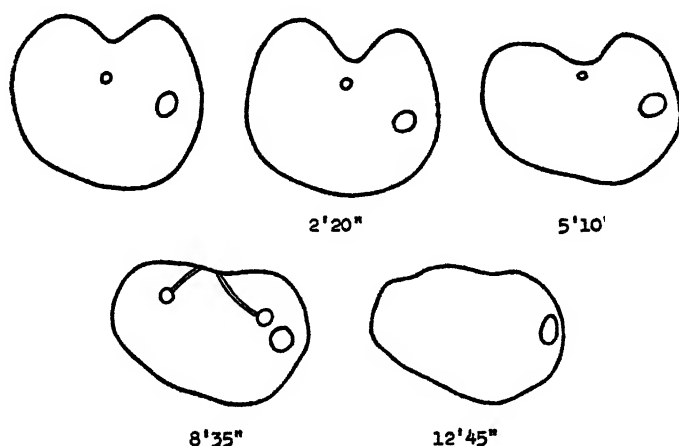


FIGURE 14. Combination of a median and a polar hole. Notice that the polar hole is not affected at all. The behavior of the median vegetal hole remains unchanged by the presence of the polar hole.

Now, if the astral rays of the vegetal region were rigid enough, this condition would immediately cause a fission in the vegetal region between the two asters as in the case of the *Astriclypeus* eggs. But, actually, the vegetal portion of *Spirocodon* eggs is much more fluid and more deformable. Therefore, instead of tearing the vegetal cortex apart (as in typical furrow formation) the cortex and the rays are both stretched out, allowing the central parts of the asters to rotate. In other words, in *Astriclypeus*, the annullment of the strain exerted by the elongating spindle is achieved by the breaking of the cortical layer (furrow formation) while in *Spirocodon* it is accomplished by the yielding of the cortex and the rays to the strain (without furrow formation).

What the authors think about the condition of the rays and the cortex is shown diagrammatically in Figure 16. The cleavage stage corresponds to stage B of Plate II. The thin lines represent extremely stretched parts of the rays and of the cortex while the thick ones are the parts which are stretched slightly or not at all. According to our interpretation, there are no rays which go to the furrow side. This ray-free narrow strip lining the furrow side must correspond to the "fan-shaped ray-free area" described in connection with *Astriclypeus* eggs (Dan and Dan, 1947). (In *Astriclypeus* eggs, as the median crossing rays are caught by the

furrow head and are pushed in as sheath rays, non-crossing rays on both sides are left behind. As a result, a fan-shaped ray-free gap appears.) In *Spirocodon*, since the arc of the fan-shaped area is stretched out so much, the shape of the fan is greatly flattened. This explains why the daughter nuclei lie so close to the furrow wall. It might be thought that data like those of Figure 16 could be obtained directly by the kaolin method. However, this is impossible since no particles can stay attached to *Spirocodon* eggs.

As the division process approaches its completion and the soft vegetal region is stretched out more and more, the central portions of the asters are released from the strain and regain their former condition. This is expressed as the rounding

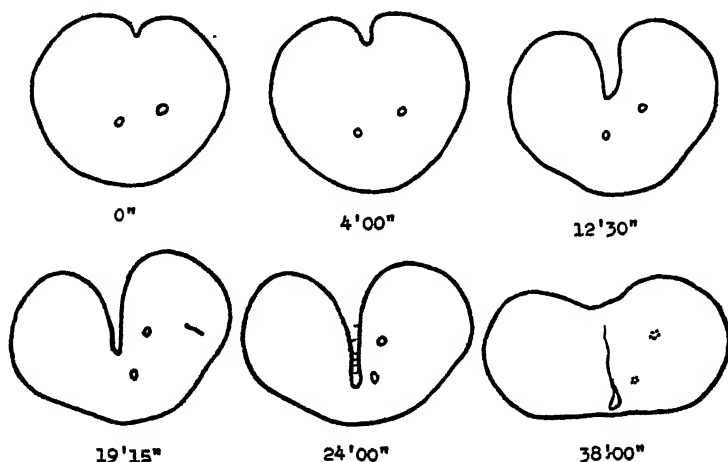


FIGURE 15. Two vegetal holes. The furrow cuts in on one side of the holes. In spite of the fact that the furrow passes quite close to the holes, no change is brought about in their shape. They vanish later. Note also that the direction of the line connecting the two holes is nearly horizontal in the beginning, becoming almost vertical after cleavage is complete. This shows that vegetal cytoplasm before cleavage comes around to the side during cleavage.

up of the incipient blastomeres in later stages of the division process. Moreover, such an astral rotation will bring about the rotation of other cell constituents. This must surely be the explanation of the movement of vacuoles and surface scars from vegetal to more animal positions.

However, the last and the most important point is that, as the vegetal material is carried around to the side and even up toward the animal pole, the relative position of the spindle within the cell will become more and more vegetal and finally the base of the U-shaped spindle will reach the vegetal extremity of the cell. In a very rough approximation, it is as if one cuts a medusan egg into two along the cleavage plane, turns the halves by  $90^\circ$  (so that the cut surfaces face downward) and pushes them together animal pole to animal pole. The cleavage is thus complete, with the nuclei in the vegetal region.

When looked at from a different angle, the above theoretical consideration brings forward two more propositions.

(1) It was stated that as the imposed strain is equalized, during the latter half of the cleavage process, the vegetal material is shifted around toward the animal side and the spindle is shifted down. These coupled movements are an action and a reaction. As a result, the authors cannot agree with the opinion that a cleavage furrow strives to reach the opposite pole by its own power.

Such an illusion must have arisen from the fact that when a cleaving medusan egg is observed, the only thing which can be seen moving is the cleavage furrow, and the cell body remains practically stationary. But this is due to a simple physical condition. When a cleaving medusan egg comes to lie on its side as the result of flattening, it must be resting on the substratum by two points, with the center of gravity of the cell somewhere on the line connecting these supporting points.

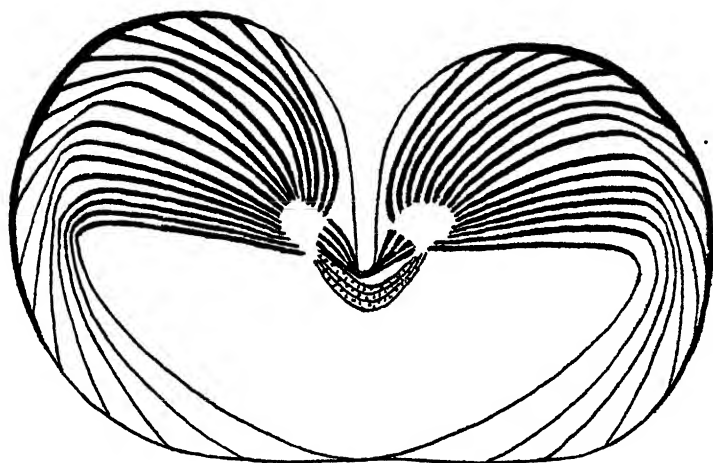


FIGURE 16. The condition of the astral rays and the cortex as interpreted by the authors from stage *B* of Plate II. The distal portions of the vegetal rays are greatly stretched. The animal cortex is drawn into the furrow and the vegetal cortex is stretched to cover the lower half of the blastomeres. Greatly stretched parts are represented by thin lines. The ray-free area is spreading along the furrow wall. This figure is only a rough qualitative representation.

Therefore, no matter what change occurs in the contour, as long as the center of gravity does not change its position, the cell cannot help remaining stationary. The rotational movement of the aster such as was discussed above will certainly not shift the position of the center of gravity very much. In other words, supporters of the opposing theory made an error in selecting the axes of coordinates. They took the more or less stationary cell contour as a reference and compared the movement of the furrow head with it. But if we have to choose a reference point, it seems to us that the position of the nuclei will be a far more reliable one. To illustrate this proposition, a series of camera lucida drawings of a dividing *Spirocodon* egg were superimposed in such a way that the nuclei would coincide as nearly as possible. The result, Figure 17, shows clearly how the vegetal material is carried up toward the animal side. If a dividing medusan egg could be suspended in some medium during cleavage, it might give the same sort of effect.

(2) The impetus for the present research was the expectation that the cause for the difference between the modes of cleavage of *Astriclypeus* and *Spirocodon* eggs was probably attributable to the difference in the degree of excentricity of the spindle. But this expectation turned out to be not strictly correct. The accurate statement must be that the difference in the mode of cleavage of the two forms lies in the difference in rigidity of the vegetal halves of the asters. Among *Astriclypeus* eggs, there is, of course, an undeniable correlation between the degree of the spindle excentricity and the degree of the onesidedness of the furrowing (Fig. 1). But this is, in turn, because there also exists a correlation between the degree of

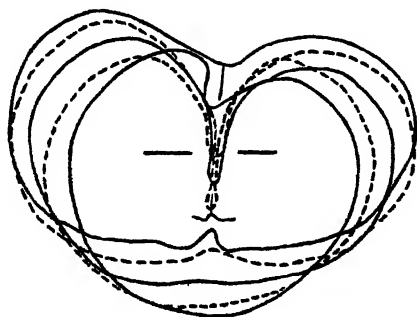


FIGURE 17. The superposition of contour drawings of a dividing *Spirocodon* egg, keeping the nuclei in as nearly a constant position as possible. But the nuclei cannot be made to overlap exactly. Their positions fall on the two lines indicated in the center. The shifting of the vegetal material to outer sides and up to the animal side is evident.

spindle excentricity and the degree of deformability of the vegetal halves of the asters.

## DISCUSSION

### *Cytoplasmic streaming and astral rotation*

Prominent among the theories which have been put forward to explain the division mechanism of medusan eggs is the view that a cytoplasmic current, considered to originate from surface tension changes, is the effective force. This theory seems to have stood partly upon the positive fact that directed movement of the cytoplasmic granules toward the tip of the furrow has been reported ever since the early days of embryology, and partly upon the negative fact that the theory has not been challenged by an alternative one. It certainly created the impression that the phenomenon of cytoplasmic streaming has special prestige in the domain of cleavage studies of medusan eggs and some investigators have even gone so far as to think that it is also true in sea-urchin eggs.

To the authors, however, there seems to exist a profound difference between the streaming in sea-urchin eggs and that in medusan eggs. The streaming in sea-urchin eggs is a current of cytoplasm toward the future cleavage plane, hence it is a current flowing prior to furrow formation (Chambers, 1938; Just, 1939, p. 284). On the contrary, the reports on medusan eggs are all concerned with cytoplasmic currents along the already formed cleavage furrow converging toward the furrow

head. Therefore, even allowing for the possibility that streaming is a chief cause of cell division, the modes of action of the two streamings must be entirely different.

Moreover, the literature on medusan cleavage is further complicated by the existence of ectoplasmic movement. As is known, the ectoplasm of *Beroë* eggs changes its thickness and usually thickens around the cleavage head (Spek, 1926). Since the ectoplasm accumulation occurs at the point toward which the cytoplasmic currents are directed, the former is often thought of as the result of the latter. But these two ought to be clearly differentiated. In short, within the vague term of "cytoplasmic streaming," three different things are included: namely, sea-urchin egg streaming, medusan egg streaming and the medusan ectoplasmic movement.

As for the streaming of sea-urchin eggs, the senior author has offered the interpretation that it is a passive movement of the fluid cytoplasm to fill in the gap formed between the separating asters. Concerning the streaming of medusan eggs, the authors are still more skeptical. They think that this streaming itself might be an illusion due to the *en masse* rotation of the asters. It must be remembered that when other investigators talk about a cytoplasmic current, what they are actually seeing is a movement of suspended granules. It is proposed that the mere fact that the granules move in reference to some landmarks outside the egg cannot have finality in the conclusion that the cytoplasm is flowing. The conclusion is allowable only when the granules move in reference to other cell structures such as the cell membrane. In the case of sea-urchin eggs, such a guarantee is provided because the streaming, after having reached the site of the future cleavage plane, is said to change its course and go inward. Since no such guarantee is available in the case of medusan eggs, who can say that it is only the cytoplasm which is moving? Furthermore, if it is a real streaming, why is there not found an accumulation of granules beneath the furrow head where the ectoplasm is thick?

According to Spek (1926), the cytoplasmic currents of *Beroë ovata* flow along the furrow sides, meet at the furrow tip and go a short distance to the inside along the median plane. This fact, he contends, is an evidence that the higher surface tension at the furrow head is causing the one-sided furrow formation. But to our surprise, on reading his paper carefully, we do not come across any streaming in the strict sense. The arrows indicating the direction of the flow in his text figures are only representing the directions of an hypothetical streaming *if such a streaming could be considered to be the cause of the movement of the green ectoplasm*. On page 60, he writes, "Während dieser Substanz Verlagerungen konnten Plasmaströmungen direkt nicht wahrgenommen werden. Konstruiert man aus dem Effekt der Plasma-verlagerungen das Strömungsbild, so erhalten wir ein eindeutiger Weise einen oberflächlichen Zustrom nach der Furche und einen axialen Abstrom von derselben Weg (S. Textabb. 3)." In other words, this is only a "Strömungsbild" where no streaming is seen. But from the authors' standpoint, the fact that his arrows are indicating the exact directions of the rotation of the asters is too significant to be overlooked.

#### *Ectoplasmic movement and aster rotation*

As for the movement of the ectoplasmic layer of *Beroë ovata*, the authors are of the opinion that this is again a passive movement. The ectoplasmic thickening at the beginning of furrow formation must be the result of the

shrinkage of the furrow surface at this stage as has been observed so many times in the kaolin experiments of sea-urchin eggs. The accumulation of the green ectoplasmic material at the points of the last connection between the blastomeres can be accounted for as follows. When the vegetal pole is stretched out and is about to be torn, the connecting bridge between the blastomeres must almost exclusively consist of the cortical material without including fluid endoplasm. Therefore, when it finally gives way, there ought to be an accumulation of the green material. During the intermediate stages, the surface area of the furrow region shrinks slightly (from unpublished data on sea-urchin eggs). Consequently, the ectoplasm around the furrow head can retain the thickness illustrated in Spek's figures.

But far more important is the later behavior of the green ectoplasm. According to Spek, in the first two cleavages, the green material which accumulates around the point of the last connection once more distributes itself evenly around the blastomeres. But at the end of the third cleavage, its re-distribution is prevented and even the nuclei are held there. As a result, at the fourth cleavage, the furrow starts to be formed at the opposite pole of the larva (the micromere pole). But this cleavage and the following one (the fifth) being extremely unequal, two sets of micromeres consisting entirely of the green material are formed. These micromeres are the mother cells of the future combs. The macromeres above them are still retaining the residue of the green material adjacent to the micromeres. In other words, as long as the cleavage is extremely unequal, the green substance stays on the same side of the larva.

After a long resting period extending for nearly two hours, the macromeres go into a division. This division is equal and the furrow starts to cut in from the micromere pole. As the furrow advances toward the other side (the polar-body pole), the last trace of the green material is carried away with it and at the completion of the cleavage, the green substance is found at the other (polar body) pole. In the following extremely unequal cleavage all the green material is given off as micromeres. As is clear, this new set of micromeres lies opposite from the group of comb-forming micromeres and they are endodermal elements.

From the authors' concept of medusan cleavage, this back and forth movement of the green ectoplasm is not surprising, since medusan blastomeres are turning somersaults at each division. When the division is unequal, although the same turning around accompanies it, the division cannot take the ectoplasmic material across the large cell and apparently leaves it in place. So it must always be an equal division which can transfer the material across to the opposite side.

In the field of amphibian embryology, the opinion is often expressed that the process of amphibian gastrulation is a formative movement by which various mesodermal elements are delivered to their definitive positions. In that sense, it is exceedingly interesting that the cleavage process itself is acting as a formative movement in *Beroë*.

The reason why the green ectoplasm re-distributes itself evenly after the first two cleavages is unknown to the authors. Spek thinks that it is due to the low plasma-viscosity during these mitoses. Whatever the explanation is, the phenomenon of the complete re-distribution of the ectoplasm seems to have a close bearing on the fact that the cleavage furrows cut in from the animal pole (the polar-body

pole) during this period. In *Spirocodon saltatrix*, on the other hand, the redistribution is prevented even for the first cleavage. As a result, the nuclei come down half way between the two poles, lying quite close to the furrow surface, and the cleavage furrow of the second division starts from there. This is functional evidence that the animal pole has come around to the furrow side and the vegetal material is spread out to cover the lower half of the blastomeres.

### *Medusan embryology and aster rotation*

The concept of the rotation of the blastomeres of medusan eggs seems to offer a hint with respect to a more general problem of the embryology of ctenophores. Among this group, it has been a puzzling fact that the egg axis and the larval axis run in opposite directions. This means that the apparent vegetal pole of the egg where the comb-forming micromeres appear becomes the fore-end of the swimming larva. This is quite contrary to the situation found in other animal phyla. For this reason, investigators who put emphasis on the later development name the two poles of ctenophore eggs exactly opposite to the way it is customarily done, calling the polar-body pole the vegetal pole (Schleip, 1929; Korschelt, 1936). Korschelt even goes so far as to illustrate the eggs up-side-down. All these confusions have their origin in the fact that people view the development of ctenophores with the conventional idea that the egg axis should coincide with the larval axis. But if the blastomeres turn upside down at each division, the egg axis changes its direction each time. In other words, among medusae, the egg axis seems to have little meaning.

### SUMMARY

1. Characteristics of division involving an excentric mitotic figure: namely, astral rotation and spindle bending, are recognizable in the division of *Spirocodon* eggs.
2. Perforation experiments indicate that although the physical condition of the animal region of *Spirocodon* eggs is similar to that of sea-urchin eggs (*Pseudocentrotus depressus*), the vegetal region is quite fluid and deformable.
3. In *Astriclypeus*, the vegetal region is like the animal in physical consistency so that the vegetal region is pulled open (i.e., furrow formation takes place) sooner or later. In *Spirocodon*, the vegetal region is quite fluid. As a result, it is never pulled open but is simply drawn out until it finally breaks. This is the fundamental difference between heart-shaped cleavage and medusan cleavage.
4. Astral rotation is discussed in connection with cytoplasmic streaming; ectoplasmic movement and medusan embryology generally.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED  
AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1947

GENERAL MEETINGS

*A delayed effect of X-rays on pepsin.* RUPERT S. ANDERSON.

It is well known that X-rays inactivate enzymes such as pepsin. Determinations of the activity remaining are usually carried out as soon as the period of irradiation is completed.

With pepsin it has been noticed that in addition to the immediate effect there is a slowly developing further inactivation. This inactivation occurs when the irradiated enzyme stands in a buffered solution at pH 5.3-5.4. Ultimately the total inactivation was several times as large as that observed initially.

It has not been possible to imitate the delayed inactivation by incubating pepsin with concentrations of hydrogen peroxide up to one millimolar. Moreover irradiated solutions which have been dialyzed against the original pure buffer solution, to restore the initial conditions as closely as possible, still show the delayed inactivation. The generality and possible significance in biological radiation experiments of this type of phenomenon will be further studied.

*On the occurrence of myoglobin in *Busycon caniculatum*.* ERIC G. BALL AND  
OCTAVIA COOPER.

In contrast to the bulk of muscle in *Busycon caniculatum*, the protractor and retractor muscles of the odontophore and the radula are bright red in color. This suggests the presence in these muscles of an iron porphyrin compound. The positive identification of this muscle pigment as an iron compound seemed of particular interest in view of the fact that this animal possesses a copper blood-pigment, haemocyanin, which displays an affinity for oxygen greater even than that of mammalian myoglobin.

A total of 86 animals yielded 74.2 grams of wet muscle. These were ground in a mortar with sand and water, the mixture centrifuged, and the turbid red supernatant brought to 60 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$ . After standing overnight the colorless precipitate was centrifuged off and the pigment precipitated from the supernatant by the addition of  $(\text{NH}_4)_2\text{SO}_4$  to 90 per cent saturation. The bright red precipitate was removed by centrifuging or filtration.

The precipitate dissolves readily in water or neutral phosphate buffer to yield a clear red solution. Its absorption spectrum shows sharp peaks at  $\lambda$  576, 540, 415, and 270  $\text{m}\mu$ , and thus resembles hemoglobin. The ratio of the extinction coefficients for 540:576 is 1.15 and for 415:540 it is 9.3. In hemoglobin the comparable ratios are 0.96 and 8.7.

The pigment reacts with  $\text{Na}_2\text{S}_2\text{O}_4$ , ferricyanide, and CO to form compounds analogous to those produced when these reagents react with hemoglobin. The CO compound shows absorption peaks at  $\lambda$  569 and 538  $\text{m}\mu$ . The span of 70 Å in the  $\alpha$  band between the  $\text{O}_2$  and CO compounds indicates a strong affinity of the compound for CO. The iron content of a salt free dry preparation is 0.17 per cent.

A crystalline chlorohemin derivative can be prepared. The absorption spectra of the ferrihemehydroxide, ferrihemecyanide, and ferrohemecyanide compounds are identical with those yielded by blood hemin.

*Physiological studies on the mechanism of action of DDT in insects.* JOHN B.  
BUCK AND MARGARET L. KEISTER.

Weight changes and oxygen uptakes were measured in adult blue bottle flies, *Phormia regina*, in high, and in low humidities, after the flies had walked on a microcrystalline surface of DDT for 15 minutes. Initial percentage body weight fractions were: water, 65; respirable substrate, 20; irrespirable material, 12.5; skeleton, 2.5. Weight loss was nearly linear in all

four groups of flies during the first 10 hours. Water loss, in per cent of original weight, totaled: dry DDT, 25.6; dry control, 14.6; wet DDT, 14; wet control, 6.5. Substrate (dry weight) loss, in per cent of original weight, over the same 10-hour period, totaled: dry DDT, 4.4; dry control, 2.4; wet DDT, 10; wet control, 1.5. Per cent substrate loss to death (18 hours for dry DDT; 8 days for wet control) totaled: dry DDT, 6; dry control, 15.2; wet DDT, 15.5; wet control, 20. Average oxygen uptake in  $\text{mm}^3/\text{hr.}/\text{mg.}$  fresh weight was 2 in both control groups, 10 in the wet DDT flies between 4 and 8 hours after poisoning, and a maximum of 7 at 4 hours in the dry DDT flies. Individual poisoned flies briefly reached peaks up to 15 times the control rate. Similar values were also reached by rare controls which were active instead of motionless. R. Q. was about 1 in the poisoned flies, 0.8 in quiescent controls. Total oxygen uptake per individual poisoned fly varied directly with both fresh and dry weight losses. Poisoned flies could accumulate oxygen debts.

Conclusions: (1) DDT enhances both water and substrate loss. (2) Low humidity reduces the respiratory stimulation caused by DDT. (3) DDT has no specific effect on either anaerobic or aerobic glycolysis, the observed respiratory and weight changes being indirect, and attributable rather to hypermotor activity. (4) Death from DDT is not primarily due to either water loss or exhaustion of respirable reserves.

*Direct determinations of internal hydrostatic pressure of Fundulus egg.* W. BUNO AND ROBERT CHAMBERS.

By the use of mercury manometer it has been possible to measure directly the hydrostatic pressure within the *Fundulus* egg which has a diameter approximately 2 mm. It was found that there is an immediate response of the egg to changes in the concentration of a balanced salt solution, namely, S. W. There is a rapid exchange of water and of salts, the exchange of salt being slightly slower. Our chief contribution is that such exchange occurs in a solution containing all the electrolytes of sea water. In preliminary experiments it was found that the addition of 5 per cent alcohol to sea water caused the pressure to fall immediately, followed by a rise to its original value and sometimes exceeding the value. This was repeated several times with similar results on the same egg in the same environment in which the concentration of the alcohol approached 30 per cent.

*Toleration of lowered oxygen tension by cave and stream crayfish.* W. D. BURBANCK, JOHN P. EDWARDS AND MADELINE P. BURBANCK.

Limestone caves are abundant in southwest Missouri and from many of them issue streams which eventually flow into the White River or its tributaries. Smallin Cave in Christian County, Missouri, sixteen miles from Springfield, is such a cave and is peculiar in two ways. First, the mouth of the cave is one of the largest in the United States measuring about ninety-eight feet wide by sixty feet high. Secondly, in the space of three-quarters of a mile, the cave tapers to less than ten feet and opens into a deep sinkhole, a condition allowing much organic material in the form of leaves and soil to enter the cave.

In Smallin Cave is found the white blind crayfish, *Cambarus setosus*. Directly outside the cave and living under practically the same conditions of temperature, dissolved oxygen, pH, and total hardness of water, lives the stream crayfish, *Cambarus rusticus*. Since the surface drainage is a factor in the total flow of water in Smallin Cave, the cave crayfish at times may live in a torrent, a swift stream, or in isolated pools. During periods of drought, the stream issuing from the cave may dry up and the stream crayfish disappear.

Following the technique of Park, Gregg, and Lutherman (*Ecology*, 21; 1940), in a series of three experiments, thirty-three stream and thirty-three cave crayfish were placed three each in liter flasks of boiled cooled water ( $\text{O}_2 = 4.0715$  cc. per liter) sealed with mineral oil. Following the death of all of the animals, oxygen analyses by the Winkler method showed that the cave and stream crayfish reduced the water to approximately the same oxygen tension—no significant difference between 0.1978 and 0.2452 cc. per liter respectively. The cave crayfish lived longer than did the stream crayfish, however. Average survival time for cave crayfish was  $829.9 \pm 35$  minutes and for the stream crayfish  $272.3 \pm 21.5$  minutes. Thus, the longer-lived cave crayfish seem to have a lower rate of metabolism than the stream crayfish.

*The shape of oil drops injected into the axoplasm of the giant nerve of the squid.*

ROBERT CHAMBERS.

The sheath of the fiber is relatively easy to puncture with a slanting thrust of the micro-pipette, the aperture of which is in the neighborhood of 1 to 2 microns in diameter. By this means it was found possible to introduce oil drops into various depths of the interior of the fiber. The oil drop assumes immediately an ovoid shape along the long axis of the nerve. Agitation of the needle inserted in the vicinity of the injection changes the consistency of the axoplasm so that the oil assumes a spherical shape. The linear arrangement of the material composing the homogenous appearing axoplasm is also indicated by subcooling the fiber to  $-3^{\circ}$  C. and inducing freezing of the axoplasm by inserting a needle. The icicles which form are cylindrical rods parallel to one another and to the long axis of the fiber. Colorimetric determinations indicate a pH of 6.8 for the normal axoplasm. When the axoplasm is liquified by damage the pH changes quickly to the neighborhood of 5.4. These experiments indicate that the axoplasm consists of ultra-microscopically arranged material, presumably in a gel state, which reverts on injury to the sol state. The pH changes observed are those of living protoplasm. This is in direct contrast with effects observed in the yolk of the *Fundulus* egg which exhibits no pH change when mechanically disturbed.

*Deformation delay in Arbacia ova centrifuged in caffeine solutions.* RALPH HOLT CHENEY.

Typically, unfertilized *Arbacia ova*, when centrifuged at  $10,000 \times$  gravity for 7 minutes, become elongated and break into halves and even quarters with more prolonged centrifugation. Sea urchin eggs in caffeine solutions resist changes in their spherical form under centrifugal force of that magnitude. For comparison, control eggs in sea water were centrifuged with eggs from the same female in 0.02 per cent, 0.10 per cent, and 2.0 per cent caffeine-in-sea-water. The response of ova when subjected to the 0.10 per cent CSW (M/200) was noted particularly because that is the concentration which has been shown by the author to definitely influence cellular metabolism.

All concentrations prevented deformation of the caffeinized eggs when centrifuged at  $10,000 \times g$ . for 7 minutes. Stratification of the echinochrome "appeared" more compact in caffeinized ova. Twelve minutes of centrifugation resulted in the breaking of 100 per cent of the controls whereas deformation and breaking in the experimental series of 0.02 per cent CSW to 2.0 per cent CSW varied from 12 per cent to 0.08 per cent of the eggs. Increasing the caffeine concentration decreased the percentage of breaking and degree of deformation.

Centrifugation at  $3,000 \times g$ . for 3 minutes resulted in no deformation of either the controls or experimentals and stratification of pigment was equal. This latter observation indicated that the "apparent" difference in stratification under greater centrifugal force, resulting in the elongation of the control eggs, was not due to any change in viscosity. It could be accounted for by the difference in the distance of sedimentation in the deformed controls and the spherical experimentals. Evidence that resistance to deformation is due to the effect of caffeine on the tension forces at the surface layers is now under investigation.

*Experiments on germinal localization in Ilyanassa.* A. C. CLEMENT.<sup>1</sup>

If eggs of *Ilyanassa* are removed from their capsules and grown in ordinary filtered sea water, they succumb to infection within a few days without attaining the veliger stage. With a semi-sterile technic, including the use of Pasteurized sea water, the naked eggs develop normally. Using this technic, the developmental capacity of various fractions of the egg has been tested. The following observations are based mainly on a study of living material.

The polar lobe is necessary for normal development. If the lobe is removed at the trefoil stage of the first cleavage, a partial larva results. Elements of the velum, including the large cilia, appear regularly and eyes are often formed. Otherwise, the larva bears little resemblance to the normal veliger, either in general form or in recognizable larval organs.

The polar lobe normally passes into the D quadrant at the second cleavage. If the D blastomere is punctured and removed, the ABC fraction produces a deficient larva resembling the

<sup>1</sup> Aided by a grant from the Carnegie research fund of the College of Charleston.

lobeless one. In contrast with this, removal of either the A, B, or C quadrant from a whole egg often results in a larva which, although showing certain deficiencies (particularly of the velum), is in most respects similar to the normal one.

If the D macromere is punctured and removed after it has given rise to three ectomeres and the mesentomere cell 4d, development is virtually normal in most cases. The only regular deficiency detected is a reduction in size of the larva. Much of the material originally contained within the polar lobe, and later the D quadrant, is removed in this operation. It thus appears that the original lobe region loses its essential morphogenetic value during the cleavage process, presumably through an allocation of potency to some or all of the first four cell derivatives of the D quadrant.

*The cytological basis of salt excretion from the gills of Fundulus heteroclitus.* D.  
EUGENE COPELAND.

*Fundulus heteroclitus* were subjected to abrupt changes from sea water to tap water and vice versa to see if the cellular mechanism for salt excretion could be demonstrated in the gills. A columnar type acidophilic cell extending from the basement membrane to the surface of the gill filament shows a secretory vesicle at the distal end in sea water and none in fresh water. The cell is rich in very fine, filamentous (possibly oriented granules) mitochondria in both sea water and fresh water. Silver nitrate tests for chloride show a very marked response in the secretory vesicle and to a less extent in the body of the cell. Fresh water adapted cells show little or no chloride. In several distilled water adapted cases, the cells rounded up and left both the free surface and the basement membrane.

*Inhibition of Arbacia egg cleavage by podophyllotoxin and related substances.*  
IVOR CORNMAN.

The effects of podophyllin upon *Asterias* eggs are reported in another abstract in this issue. Podophyllin is a mixture of podophyllotoxin (m.w. 414), quercetin (m.w. 321), podophyllinic acid, and unknown constituents. The effect of podophyllin on *Arbacia* egg cleavage appears to represent the combined effects of podophyllotoxin and quercetin, revealing a wide range of concentrations (0.4–2 mg./L) over which it slows cleavage slightly, then a sharp threshold at which cleavage is completely inhibited (4–6 mg./L). Like podophyllotoxin, podophyllin completely inhibits the cleavage of some eggs at concentrations which only moderately delay the cleavage of the remainder. Quercetin (20 mg./L) more than doubles the time required for cleavage while permitting all cells to cleave, and delays cleavage time 10 per cent at a much lower concentration (0.6 mg./L). Podophyllotoxin delays cleavage time 10 per cent at 0.07 mg./L and blocks completely at 0.5 mg./L. Thus these two compounds are more active than colchicine (m.w. 399), which acts at 10–80 mg./L. Rutin, which contains a quercetin nucleus (m.w. 664), delays cleavage very slightly at saturation.

Other visible processes, e.g., activity of sperm and lifting of the fertilization membrane, proceed normally in podophyllin at concentrations as high as 100 mg./L. The cleavage furrow is unaffected, as evidenced by cleavage of eggs which have reached anaphase at the time of exposure to 0.5 mg./L of podophyllotoxin.

Two other echinoderms are more sensitive to podophyllotoxin than is *Arbacia*. The maturation of *Asterias* eggs is blocked completely at 0.01 mg./L. Cleavage of *Echinarachnius* eggs is slowed at 0.01 mg./L and blocked completely at 0.04 mg./L.

*The distribution of phosphorus in the unfertilized egg of Arbacia punctulata.*  
ROBERT K. CRANE.

Mature, unfertilized eggs from freshly collected *Arbacia* contain a total of 130 milligrams phosphorus (Fiske and Subbarow) per gram nitrogen (Folin), or 0.31 milligram per cent of wet weight.

Preliminary partition (expressed in milligrams phosphorus per gram nitrogen): *acid-insoluble*, 35 (range 31–39); *acid-soluble*, 53 (range 47–60); *ether-soluble*, 53 (range 40–66). Separate samples were used for each fraction.

Fractionation of acid-insoluble (Schmidt and Tannhauser, *Jour. Biol. Chem.*, 161; 83, 1945): phosphoprotein, 27; ribonucleic acid, 65; and desoxyribonucleic acid, 15.

Analysis of total acid-soluble was made on trichloroacetic acid filtrates. Since echinochrome interfered with analysis of orthophosphate, an ammonium sulfate extract (Lowry and Lopez, *Jour. Biol. Chem.*, 162; 421, 1946), free of echinochrome and proteins, was prepared. This yielded 35 milligrams phosphorus per gram egg nitrogen from suspensions which had total acid-soluble of 50; of the 35, 11 were inorganic (Lowry and Lopez), 14 were liberated by 7-minute hydrolysis in 1 N hydrochloric acid at 100 degrees centigrade, 10 more by 100-minute hydrolysis. Total content cannot be analysed in ammonium sulfate solutions.

For unfertilized eggs, a nitrogen ratio of 100 milligrams per gram dry weight has been reported. Suspensions in July (fresh urchins) confirmed, but those prepared in August (stored urchins) showed ratio as low as 55.

Total phosphorus ratio (dry weight) increased with lower nitrogen. At nitrogen ratio of 55, total phosphorus approximated 17 instead of 13 milligrams per gram dry weight. Significant changes also occurred in fractions.

Logarithmic curves relating total and fractions of phosphorus to nitrogen (dry weight basis) yielded straight lines. Extrapolation to 100 milligrams nitrogen per gram dry weight showed 13 milligrams total phosphorus per gram dry weight ( $0.1 \times$  value based on nitrogen). Data were insufficient for similar derivation of fractions. In the future, data will be thus graphically analyzed in belief that truer values than afforded by averages will be obtained.

The author worked in the laboratory of Dr. Clowes and is grateful for many helpful suggestions

*A preliminary study of the vertical distribution of marine organisms in the Cape Cod area.* ELIZABETH M. FAHEY.

Intertidal marine organisms appear at low tide to be arranged in a number of superposed zones. Transects across these zones from the highest levels in which marine organisms appear to the lowest levels to which the tides recede have been studied, with the vertical distribution of the organisms found thereon recorded. Though the overall heights of the transects vary with the mean tide range from place to place, the data gathered from Plymouth, Provincetown, Lewis Bay (Hyannis), Nobska Point, Penikese Island and in Woods Hole correspond well as to the number of zones and in their approximate proportion to the height of the transect. Likewise, from place to place, these zones have had, at the same time of the season, about the same floristic and faunistic constitutions and the same positions in relation to each other.

Data from a study of the repopulation of denuded transects at Nobska Point support the hypothesis that the organisms "seed" onto the rocks throughout a greater vertical range than the same species occupy as adults. The primary repopulating macroscopic forms were *Littorina littorea* and species of *Calothrix*, *Enteromorpha*, and *Polysiphonia*.

*Interaction between the implanted embryos and the internal organs of an adult teleost, Opsanus tau.* PAUL S. GALTISOFF AND EUGENIA GALTISOFF.

Embryos of *Opsanus tau*, from 3 to 5 mm. long, introduced with aseptic precautions intra-peritoneally into an adult toad fish survive and attach themselves to the abdominal wall, liver, intestines and possibly to other organs. Normal development of the implanted embryo ceases, but the embryonic tissues continue to grow in an erratic manner forming teratomas. Embryos attached to the surface of the liver produced metastases which invaded and destroyed the underlying portions of this organ. Three week old tumors, growing in the liver, consisted of easily recognizable elements of connective tissue, blood, notochord, cartilage, and muscle fibers. No traces were found, however, of the epidermis and typical mucus cells present in the normal embryos.

One encapsulated teratoma, attached to the peritoneum of the abdominal wall, produced no metastases. It consisted of various embryonic tissues and organs irregularly scattered within the capsule.

None of the eight fishes used in the experiments died as a result of the operation. The behavior of the operated fishes appeared to be normal.

From a preliminary study of the experimental material conclusion is drawn that the regulatory mechanism responsible for the orderly development of the embryo ceases to function when the latter is implanted in the organs of an adult organism. Erratic growth of some of the embryonic tissues continues, however, and results in the destruction of the cells and tissues of the host.

*Altering the expression of position effect.* W. GOODSMITH AND TAYLOR HINTON.

Plum eye color in *Drosophila melanogaster* is due to a complex inversion in the second chromosome. This "position effect" when crossed to most stocks does not vary in expression. However, when crossed to Inversion (2R)NS, offspring appeared with reddish, almost wild-type eyes and exceptional mottling of the pigment. An attempt was made to ascertain whether it was the inversion causing the modification of Plum or whether it was some other factor in that stock. Therefore, Inversion (2R)NS was extracted from the original modifying stock and incorporated into a new stock containing Inversion (2L)t, which does not modify Plum. This stock, In(2L)t, In(2R)NS/Ins(2L+2R)Cy, was crossed to Plum and the majority (906) of the offspring could be placed into 2 classes both phenotypically and cytologically. These were: Ins(2L+2R)Cy/Plum, giving a normal Plum eye color, and In(2L)t, In(2R)NS/Plum, giving a modified expression of Plum as previously described. Two additional types were produced (34 individuals): one, a normal Plum without the Curly phenotype, and the second, a modified Plum with the Curly phenotype. Cytological analysis has shown these to be In(2L)t, In(2R)Cy/Plum, and In(2L)Cy, In(2R)NS/Plum, respectively. Therefore it can be concluded that the modifying effect is correlated with the presence of the (2R)NS inversion. It is conceivable that it is not the inversion *per se* but rather genetic modifiers within the inversion which are responsible for the effect. However, a series of chromosomal rearrangements recently obtained by irradiation include 3 which have the same effect upon Plum as does In(2R)NS. It would be too coincidental that in all of these a genetic modifier mutated simultaneously with the occurrence of a chromosomal rearrangement. Therefore, it is suggested that in several cases a chromosomal rearrangement has modifying effect upon another chromosomal rearrangement. Tentatively, this might be termed a "position effect upon a position effect."

*Conditions affecting development of the male phase in Crepidula plana.* HARLEY N. GOULD.

In previous studies the author concluded that development of the adult male phase in the sexual cycle of this species necessitated close association, but not contact, of the small potential males with larger individuals of the same species. W. H. Coe has since stated, however, that "neither in *Crepidula plana* nor in any of the other five species of that genus studied is association with other individuals a prerequisite for the development of the functional male phase." To test this matter further, very young spat of *Crepidula plana* of one to five millimeters shell length which had not yet developed any external indication of sex were removed from the clusters growing in hermit crab shells and allowed to live in glass dishes with running sea water, free from any influence of larger individuals.

Over 400 such specimens have been examined at ten-day intervals. Over 100 have to date been followed for more than forty days. Many have attained and passed the average size of males. No adult males have appeared in any of the cultures. An incomplete development of maleness, indicated externally by a stumpy rudiment of the penis, may appear transiently, usually in about thirty days and in the case of animals of five to seven millimeters shell length. Individuals which have reached a shell length of fourteen or fifteen millimeters after forty days culture are of a size where female development may be under way. The condition of the gonads in this material will be reported later when microscopic sections are made.

*The effect of sulphhydryl inhibitors on the anaerobic glycolysis of scallop and thyone muscle.* J. HARTING.

Steinbach and Kawata (*Federation Proc.*, 3: 46; 1946) reported that iodoacetate accelerated the rate at which a cut surface of scallop muscle changed to restore the normal membrane con-

ditions, as indicated by measurements of the injury potential. Since iodoacetic acid is known to inhibit metabolic processes by combining with the  $-SH$  groups essential for enzyme activity, it was decided to investigate the effect of the sulfhydryl inhibitors on the glycolysis of scallop muscle. The striated portion of the scallop muscle was teased into thin strips and suspended in van't Hoff's artificial sea water, containing 0.0238 M  $NaHCO_3$ , 0.01 M glucose, and saturated with  $N_2:CO_2$  (95:5), pH 7.2. The rate of glycolysis was measured manometrically at 26° C. with  $N_2:CO_2$  as the gas phase. When the alkylating agents, iodoacetate (0.001 M) and iodoacetamide (0.005 M), were added twenty minutes before manometric readings started, there was inhibition of glycolysis ( $CO_2$  output) about forty minutes later. When they were added one hour after the measurement of glycolysis started, there was a steady increase in  $CO_2$  output, which continued for one to two hours. In contrast to scallop muscle, the glycolysis of thylene muscle was inhibited by iodoacetate whether it was added before or during the measurement of glycolysis. Iodosobenzoate (0.001 M), an oxidizing agent, and p-chloromercuribenzoate (0.001 M), a mercaptide-forming compound, produced an immediate increase in the glycolysis of scallop muscle. The increase in  $CO_2$  evolution in the case of these reagents was independent of the time at which they were added. p-chloromercuribenzoate (0.001 M) also had a stimulating effect on thylene muscle.

*On the relation between oxygen consumption, fertilization membrane formation, and cell division in artificially fertilized Arbacia eggs.* A. K. KELTCH AND G. H. A. CLOWES.

Oxygen consumption of sperm fertilized *Arbacia* eggs is about 2.7 times that of unfertilized eggs under present conditions of experiment. This investigation is to determine (1) whether the same ratio obtains for artificially fertilized eggs and (2) whether any such increase in oxygen consumption is associated with (a) formation of a fertilization membrane or (b) subsequent cell division, or both.

Artificial parthenogenesis was accomplished by exposing unfertilized *Arbacia* eggs for 25 minutes to sea water to which sodium chloride had been added to give a concentration equivalent to 1.05 M NaCl. The egg-containing hypertonic solution was then diluted with 30 volumes of sea water, the eggs allowed to settle, the supernatant fluid syphoned off, and the egg suspension once more diluted with 30 volumes of sea water, allowed to settle, decanted, and the eggs taken to a known volume of approximately 2 per cent. Equivalent concentrations of sperm fertilized eggs were subjected to the same series of procedures. Counts were made on both artificially and sperm fertilized egg suspensions, equivalent amounts were put in Warburg vessels for two

TABLE I  
*Oxygen consumption and per cent division of Arbacia eggs*  
(A) sperm fertilized and (B) artificially fertilized

(From previous work the oxygen consumption of unfertilized *Arbacia* eggs is from 1.5 to 1.7 c. mm.  $O_2$  per hour per 10 c. mm. eggs.)

Exp. No.	c. mm. $O_2$ per 10 c. mm. eggs per hr.		Per cent of eggs divided in $3\frac{1}{2}$ hrs.	
	A	B	A	B
23 W	4.8	3.6	98	45
24 W	4.7	4.3	98	45
25 W	4.4	3.8	98	24
28 W	5.9	6.0	98	24
29 W	5.1	5.3	98	26
30 W	5.3	5.5	98	18
31 W	5.2	5.0	98	24
Average	5.1	4.8		

In all cases the eggs gave 98 to 100 per cent fertilization membranes.



hours to determine oxygen consumption, and samples were removed to determine extent of cell division.

The following table indicates that oxygen consumption was approximately the same for artificially and sperm fertilized eggs. This is extremely interesting in view of the fact that while in both the artificially and sperm fertilized eggs membrane formation was from 98 to 100 per cent, recognizable cell division never exceeded 45 per cent and was as low as 18 per cent in one set of artificially fertilized eggs. It appears, therefore, that the increase in oxygen consumption is associated with some change of condition of the eggs when the fertilization membrane has been formed and is not dependent on subsequent cell division.

*Effect of dinitrocresol on oxygen consumption and cell division in artificially fertilized as compared with sperm fertilized Arbacia eggs.* A. K. KELTCH, C. P. WALTERS, AND G. H. A. CLOWES.

Certain substituted phenols stimulate oxygen consumption of sperm fertilized Arbacia eggs and, at concentrations giving optimum oxygen consumption, reversibly block cell division. This investigation is to determine whether the same effect is exerted on oxygen consumption and division of artificially fertilized eggs.

Artificial parthenogenesis was accomplished as described in previous paper except that after 25 minutes in hypertonic solution sample egg suspensions were diluted twice with 30 volumes sea water or sea water containing desired concentrations dinitrocresol before bringing egg suspensions to 2 per cent concentration. Equivalent samples of sperm fertilized eggs were subjected to the same series of procedures. Egg counts were made and two-hour oxygen consumption determined for artificially and sperm fertilized series in sea water and various con-

TABLE I

*Stimulation by 4,6-dinitro-o-cresol of oxygen consumption of Arbacia eggs fertilized by (A) sperm and (B) artificial parthenogenesis*

The figures presented in the table represent values of the ratio:

$$\frac{\text{oxygen consumption of treated eggs}}{\text{oxygen consumption of control eggs}} \times 100.$$

Molar concentration DNC  $\times 10^{-4}$

Exp. no.	0	4	8	16	32	64
A	23 W	100		253	172	134
	24 W	100		246	179	160
	25 W	100		236	151	119
	28 W	100		213	159	126
	29 W	100		182	152	134
	30 W	100	173	217	230	
	31 W	100	216	234	222	
	Average	100	194	225	226	163
B	23 W	100		201	175	
	24 W	100		266	214	178
	25 W	100		161	132	120
	28 W	100		197	144	109
	29 W	100		170	152	120
	30 W	100	155	199	195	
	31 W	100	200	227	206	
	Average	100	187	213	214	163

centrations of dinitrocresol, counts made to determine extent of division, and samples returned to sea water to ascertain extent of recovery.

The accompanying table shows that various concentrations of 4,6-dinitro-o-cresol gave approximately the same percentage increase of oxygen consumption in artificially and sperm fertilized eggs. Furthermore, the block to cell division occurred at the same concentrations of 4,6-dinitro-o-cresol and also of 2,4,6-trichlorophenol in artificially and sperm fertilized eggs. However, when returned to sea water after three hours' exposure to reagents, sperm fertilized eggs showed 100 per cent recovery in all concentrations, whereas artificially fertilized eggs exposed to higher concentrations developed only to an abnormal, many-cell stage.

*Urea excretion in the smooth dogfish.* RUDOLF T. KEMPTON AND BURTON STECKLER.

Study of urea reabsorption by the kidneys of the smooth dogfish has yielded preliminary data indicating the magnitude of the reabsorptive process. As is well known, the elasmobranchs utilize high levels of blood urea as a mechanism of keeping their blood isotonic with sea water. The reabsorption of urea is a fundamental part of the method of maintaining this high blood level.

Inulin clearance was used as a measure of glomerular filtration. Simultaneous urea clearance permits the calculation of the behavior of kidney in relation to urea as well as to water.

In the series of experiments the plasma level of urea (plus ammonia) nitrogen averaged 961 mgm.%, the equivalent in urea of more than 2 per cent. Plasma rather than whole blood determinations were made due to the impermeability to urea of the red cells of this species. Urine urea (plus ammonia) levels ranged from 24 to 73 per cent (average 48 per cent) of the plasma concentration. This fact gives clear indication that urea reabsorption is due to the activity of cells rather than to a passive back diffusion.

While from 27 to 74 per cent (average 60 per cent) of the water in the filtrate was being reabsorbed, from 72 to 92 per cent (average 82 per cent) of the filtered urea was reabsorbed. The data thus far do not indicate any clear correlation between the two processes.

Calculations of the amount of reabsorption per 100 ml. of filtrate yield a comparatively constant value, ranging from 710 mgm. to 922 mgm. (average 808 mgm.).

On the basis of these and earlier data on water reabsorption it is provisionally concluded that under the experimental conditions there is considerable variation in the number of active nephrons, both in different animals and in the same animal at different times. Further experiments are to be conducted to determine the limits of urea reabsorption and to determine whether this process is related to the reabsorption of other materials such as water and reducing sugar.

*The transient response of luminous bacteria to sudden temperature increments and related experiments on drug action and protein denaturation.* G. L. KREEZER AND E. H. KREEZER.

In a linear physical system, the determination of its transient response to a sudden disturbance supplies information about the internal organization of the system not obtainable from static measurements. The transient response refers to that portion of the response during which the system is changing from a preceding steady-state to that following the disturbance. The present experiments were undertaken to test the usefulness of such determinations of transient response in non-electrical biological systems. Suspensions of luminous bacteria (*Achromobacter fischeri*) were subjected to sudden increments of temperature, and the rate of temperature rise and the associated rise in the brightness of the suspension recorded with amplifier-oscillograph equipment. These records show a definite lag in the luminescent response behind temperature. Supplementary experiments and computations indicate that the lag cannot be attributed to a lag in heat transmission through the suspension, or through the bacterial cell membrane. It seems necessary to attribute it to a lag occurring in the chain of chemical reactions underlying bioluminescence. Various chemical hypotheses are considered.

Preliminary experiments were also performed on the transient decay of luminescence associated with the sudden application of various drugs (ethyl alcohol, urethane, sulphamylamide). Transient response curves were obtained with time constants of the order of fractions of a second.

The results thus suggest that this procedure may provide a useful method for measuring the rate of action of drugs on living cells.

In order to investigate similar phenomena in purely chemical systems, protein solutions were selected as offering suitable material. Preliminary experiments upon dilute solutions of serum albumin show that sudden increments of temperature produce an immediate and progressive increase in the turbidity of the solution, followed by apparently complete reversal on cooling. Further experiments on the "denaturation response curves" of different proteins, and the response to different denaturing agents are in progress. (These experiments were aided by a grant from the Penrose Fund of the American Philosophical Society.)

*The effect of sinus gland extract on certain enzyme systems.* ELOISE KUNTZ.

The endocrine function of the crustacean sinus gland has been well established by numerous studies of its diverse effects on intact animals. This is a preliminary report on the effect of sinus gland extract on enzyme systems.

Extracts were prepared by suspending the glands in water, crushing them and then freezing. Several hours before use the extract was thawed and stored in the refrigerator. The animals used were lobsters, *Homarus americanus*, and the spider crabs, *Libinia dubia* and *L. emarginata*.

The ability of tissue homogenates to utilize 2-6-dichlorophenol indophenol as a hydrogen carrier was measured and found to be no greater in the presence of sinus gland extract. One of the principal cellular constituents capable of reducing this dye is glutathione. The addition of an extract of sinus gland doubled the reducing ability of glutathione. Sinus gland extract alone did not reduce the dye. This suggested that the hormone may serve as a hydrogen carrier by reducing active groups such as sulfhydryls.

The coupled oxidation-phosphorylation by a homogenate of *Libinia* ventral nerve ring from eyestalkless animals has also been studied. In a system containing homogenate, adenylic acid, glutathione, arginine and inorganic phosphate the oxidation of Na succinate was used to supply the energy for the formation of arginine phosphate. This system binds inorganic phosphate in the presence of oxygen. The rate of phosphate uptake is enhanced by the presence of sinus gland extract. In the absence of glutathione or oxygen organic phosphate is hydrolyzed to phosphoric acid. In this reaction, too, the addition of sinus gland extract accelerates the breakdown of phosphate. These experiments indicate that sinus gland extract acts as an accelerator in both cases, but it apparently does not affect the direction of the reaction.

*Germ cell formation of Tubularia crocea with special reference to the germ plasm theory.* C. K. LIU.

The formation of the male and female germ cells of the hydroid *Tubularia crocea* has been studied with a view to test the germ plasm theory. In contradiction to Weismann's hypothesis, the germ cells are of endodermal origin instead of ectodermal. No interstitial cells are involved in their formation, and there is neither early segregation nor migration of germ cell. The entocodon is formed by an apical proliferation and subsequent delamination of the endoderm of the gonophoral bud, which is nothing but an extension of the body wall of the hydranth. From the entocodon the germ mass is differentiated. The latter, however, has been found to develop not only into germ cells but also into scattered septum cells, which are not germinal, but somatic. The "germ plasm" therefore is originated from somatic cells and in turn gives rise to somatic cells in addition to germ cells. Regeneration experiments further indicate that the general somatic cells throughout the length of the stalk have the potentiality of forming functional germ cells, male or female. All these go to prove that the "germ plasm" and soma remain permanently mixed with each other in the stalk tissue, and such a distinction is far-fetched and meaningless. The result of this study is therefore incompatible with the germ plasm theory.

*Observations on the hemolytic action of urea and sodium salicylate.* LOIS H. LOVE, W. E. LOVE AND M. H. JACOBS.

It has been suggested by Wilbrandt that the primary action of certain hemolytic agents on erythrocytes is a denaturation of proteins of the cell surface, resulting in a permeability to

cations followed by osmotic hemolysis, through the operation of the Donnan equilibrium. Observations were accordingly made on the hemolytic action of two agents known to be efficient protein denaturants, namely, urea in high concentrations and sodium salicylate. In general, both substances proved to be much more difficult to work with than butyl alcohol and sodium taurocholate, the behavior of which is discussed elsewhere. Part of the difficulty was due to the production of internal changes in the cells, which sometimes very obviously affected their osmotic properties; part of it was due to the smallness of the difference in the exposures required to produce permeability to Na ions, on the one hand, and to sucrose molecules, on the other. With sufficient patience, however, it was possible with both agents to prepare cells which showed several of the osmotic characteristics of cation-permeable erythrocytes already discussed, namely, (1) hemolysis in NaCl and preservation in sucrose solutions, with a marked protective action of sucrose in mixed solutions, (2) a minimum rate of hemolysis in NaCl solutions at a pH near the isoelectric point of hemoglobin, and (3) a strong and rapid shrinkage of cells treated in NaCl or a mixture of NaCl and sucrose on being placed in pure sucrose isosmotic with blood. The shrinkage in a sucrose solution of this concentration was several times observed to restore to visibility erythrocytes that had already swollen to the point of invisibility, thereby giving rise to a type of "reversible hemolysis" somewhat different from the common one in which shrinkage is produced by a sudden increase of the osmotic pressure of the external solution.

*Effects of thiourea and phenylthiourea upon the development of Plethodon cinereus.*  
W. GARDNER LYNN.

The Red-backed Salamander is unusual among amphibians in that it lays large-yolked, terrestrial eggs and has no aquatic larval stage. During development certain larval characters, such as external gills, appear transiently but these degenerate before hatching and the young salamanders emerge from the egg with the adult body form. In view of the well-known importance of the thyroid hormone in the metamorphosis of the larvae of ordinary amphibians, a study of the rôle of the thyroid in the precocious metamorphosis of *Plethodon* was undertaken through the use of thyroid-inhibiting drugs.

Fifteen clutches of eggs were collected between July 2 and July 14 in rotting logs in the Devil's Lane Tract near Woods Hole. At the early limb-bud stage the jelly layers and vitelline membranes were removed and, for each clutch, some eggs were kept in tap-water while others were kept in 0.01 per cent thiourea or in 0.005 per cent phenylthiourea. Control animals developed normally and underwent gill resorption and other metamorphic changes on about the 25th day. Experimental animals in both solutions retained the gills, failed to shed the larval skin and showed no differentiation of eyelids as long as treatment was continued. Cessation of treatment was followed by metamorphosis; gill resorption beginning within three days. It appears that, in *Plethodon*, the later stages of intra-oval life constitute a metamorphosis which is under thyroid control. By inhibition of the thyroid the metamorphic changes can be prevented and an "aquatic larva" is thus produced in a salamander which normally has no such stage.

An additional effect of phenylthiourea was a depigmentation of the embryo involving both the skin and the pigmented coat of the eye so that animals kept in this drug were entirely without color. This effect is probably to be attributed to failure of melanin production through inhibition of tyrosinase.

*Are —SH groups involved in the penetration of glycerol into human red cells?*  
A. K. PARPART, E. S. G. BARRON AND T. DEVY.

The apparent rate of penetration of glycerol into human red cells, as studied by hemolysis, is delayed by a number of compounds that inhibit the action of —SH groups (parachloromercuribenzoate; Hg; chlorpicrin; Cu). Other inhibitors have no such effect (paracarboxyphenylarsenoxide; Cd; iodosobenzoate; iodoacetamide). Volume change methods for the study of the penetration of glycerol did not show any inhibition, except in the case of Cu. It is suggested that those inhibitors which are active do so through their action on the outward diffusion of hemoglobin in the hemolysis studies. Human red cells exposed for 30 minutes to parachloromercuribenzoate also show a delay for water and ethylene glycol hemolysis; a further confirmation of the above suggested mode of action.

*A comparison of the energy requirements of the sand dollar and sea urchin eggs.*  
W. A. ROBBIE.

With respect to maintenance and the process of cleavage, marine eggs may be classified into three groups: (1) those that demand respiratory energy for both maintenance and cleavage; (2) those that need oxygen for cleavage but will survive without it; and (3) those that will both divide and survive under anaerobic conditions. These metabolic characteristics of the developing egg may be studied by experiments at low oxygen tensions or by cyanide inhibition of the heavy metal enzyme systems responsible for respiration.

The sand dollar, *Echinarachnius parma*, is a good example of the type of egg that requires an aerobic system for both cell division and maintenance. *Arbacia* represents the second type, and *Fundulus* will survive and cleave in cyanide or in the low-oxygen bottom mud which is its usual environment. Experiments on *Echinarachnius* at the Mount Desert Island Biological Laboratory and on *Arbacia* at Woods Hole show that respiration and cell division are inhibited in both by low concentrations of cyanide. The striking difference is in the irreversibility of inhibition of respiration in the sand dollar egg, whether by oxygen lack or cyanide. One-half hour in one ten-thousandth molar cyanide results in eventual cytolysis of most of the eggs, whereas *Arbacia* will withstand 24 hours with no apparent ill effects. The coincidence of this cytolysis with the point of marginal inhibition of respiration by cyanide suggests that a metabolic factor is involved.

The difference in the behavior of the *Echinarachnius* and the *Arbacia* egg may be due to the activity of a glycolytic system, since it has been shown that *Arbacia* eggs in cyanide produce lactic acid. This glycolytic process perhaps provides enough energy to maintain the egg during the period of respiratory inhibition. Glycolysis measurements on *Echinarachnius* have not yet been made.

*A new peritrich from Woods Hole.* M. A. RUDZINSKA.

During the month of August, while making daily inspections of material from live cars immersed in the Eel Pond, I found a peritrichous ciliate not yet described. Observations were limited due to the fact that only three individuals were found.

These three ciliates, about 0.2 mm. long, were attached to a tiny smooth stick with transverse stripes. The body form is cup-like and narrower at the anterior and posterior ends. The anterior region is conspicuously ciliated with one ring of long cilia which surround a very large cytostome. The cilia are in continuous movement. There is one contractile vacuole located near the cytostome. The posterior end has two pseudopodia like lamellae by which it is attached to the stick. The body is not contractile.

These forms have a very unique movement along the stick. They go from one end of the stick to the other, stopping when they reach the end of the stick and then returning the whole length again. The form of the body does not change during this movement, nor do any contractions of the lamellae, by which they are attached, occur when they move. The motion is like a smooth, constant sliding. When removed from the stick they affix to the slip and stop moving. Only the cilia of the cytostome retain their movements.

The above described, very characteristic motion was also observed by Dr. A. A. Schaeffer, and Dr. W. D. Burbanck. It is interesting to note that the individuals seen by these men were attached to the same sort of stick that I have described.

It seems to me that the described peritrich belongs to the family Scyphididae Kahl because the posterior end is without stalk and they stick to objects by attaching lamellae. It is a new genus and a new species.

I only had this material for a short time, and did not make stained preparations. More details of the construction of the body will help to explain the unusual type of movement.

*Androgenetic males and X-ray induced cytoplasmic injury.* ANNA R. WHITING.

Unlaid eggs of wild type *Habrobracon juglandis* females X-rayed in first meiotic metaphase (lethal dose 2,200 r), if unfertilized, develop into wild type haploid gynogenetic males; if fertilized by untreated sperm with recessive factors they develop into wild type diploid females

or recessive haploid androgenetic males. Unlaid eggs X-rayed in first meiotic prophase (lethal dose 48,000 r) behave in the same manner except that no androgenetic males are produced. Androgenetic males appear to arise through retardation of the egg pronucleus by chromatin bridges, numerous after treatment of metaphase I. These bridges are formed but rarely after treatment of prophase I and, when present, are not numerous enough to retard female pronucleus. In spite of the extreme sensitivity of metaphase I eggs, androgenetic males develop in them when they are irradiated with doses up to lethal for prophase I, about twenty-two times their own lethal dose. At doses above this none has been produced. Their number decreases as the prophase I lethal dose is approached. This indicates cytoplasmic injury at high doses. Two factors, then, are involved in development of androgenetic males, (1) injury to the egg chromosomes favoring their production, (2) injury to the cytoplasm inhibiting it. The dose at which these two effects "cross," marked by highest production of androgenetic males, is about 12,000-15,000 r. At about 48,000 r cytoplasmic injury completely dominates the former.

*Action of X-rays on mating types and conjugation of Paramecium bursaria.*  
RALPH WICHTERMAN.

Opposite mating types of the green *Paramecium*, *P. bursaria*, highly reactive for the mating reaction were irradiated in celluloid boats at dosages ranging from 100,000 to one million roentgens. Irradiation affects the mating reaction, destroys the zoochlorellae and delays and lengthens conjugation.

When opposite mating types are irradiated up to 300,000 r and mixed, only small clumps of usually less than a dozen paramecia are formed instead of the large clumps of a hundred or more shown in the controls. The mating reaction does not result in the immediate formation of joined pairs in conjugation after irradiation. However on the day following irradiation, small clumps are formed again which then result in conjugating pairs for those irradiated up to 200,000 r; a second day must elapse for conjugation to occur in those irradiated with 300,000 r. When paramecia of one mating type are irradiated up to 300,000 r and mixed with unirradiated paramecia of the opposite mating type, clumping occurs similar to that of the controls but does not result in conjugation until the following day. When both mating types are irradiated with 400,000 r and 500,000 r respectively and mixed, the mating reaction is slower and the number of paramecia in clumps smaller. The mating reaction is temporarily stopped with 600,000 r, but small clumps are formed three days after irradiation. In these higher dosages, the conjugation lag increases; with mating types irradiated with 600,000 r, conjugating pairs are not formed until 12-14 days after irradiation although clumping occurs earlier.

Animals of opposite mating type when irradiated with 700,000 r and then mixed show no mating reaction but do so when mixed with unirradiated paramecia of opposite type. At this dosage and up to one million roentgens paramecia live for a time but do not survive. At 600,000 r and less, many paramecia not only survive but after a lag period have a fission rate similar to the controls.

A method is now available for obtaining colorless, zoochlorellae-free races of *P. bursaria* which occur with rarity in nature. Dosages of 300,000 to 600,000 r destroyed the zoochlorellae and when the irradiated specimens were isolated, new clones of zoochlorellae-free paramecia were obtained. After two weeks, these clones of irradiated and colorless *P. bursaria* of a given sex type were mixed with typically green specimens of opposite sex type. Mating and conjugation followed. The sex type of irradiated zoochlorellae-free paramecia was the same as before irradiation.

Unirradiated paramecia not only survive but reproduce in clone cultures which had been irradiated with one million and less roentgens, hence such fluid does not appear to be toxic to the organisms.

There is clear evidence of a difference in susceptibility to roentgen rays in the mating types tested.

Irradiated conjugants (300,000 r) remain together much longer in sexual union than those of the controls; a small percentage are unable to survive.

Dosages of 100,000 r stimulate activity of the paramecia but higher ones progressively retard locomotion, markedly increase the viscosity of the cytoplasm and produce small, clear vesicles (hyaloplasm) on the pellicle which then pass into the culture medium.

*Effect of temperature on light absorption by the cell-free coelomic fluid in Phascolosoma gouldii.* CHARLES G. WILBER AND RUTH P. ALSCHER.

Very little is known concerning the physical characteristics of invertebrate body fluids. In an attempt to ascertain the relationship of invertebrate fluids to those of higher animals, a series of experiments was made to test the effect of heating on the absorption of light by the cell-free coelomic fluid in *Phascolosoma gouldii*. Coelomic fluid was removed from the worms, centrifuged, and the cell-free supernatant fluid sealed in carefully matched soft glass test tubes. Some of the tubes were heated for 5 minutes in a carefully controlled water-bath at temperatures from 24° C. to 90° C.; others were heated at similar temperatures for 20 minutes. The absorption of light by the heated fluids was compared with that of distilled water in an electric photometer. If the value recorded on the photometer is plotted against temperature, evidence of a decrease in light absorption at about 40° C. followed by an increase beginning at 50° C. is obtained. A graph obtained by plotting optical density against temperature indicates that the density decreases at about 40° C. but suddenly increases rapidly at about 50° C. Apparently, there are 2 critical temperatures for the cell-free fluid in *Phascolosoma*: (1) 40° C. above which heating brings about a decrease in particle size, with resulting decrease in light absorption; (2) 50° C. above which particle size is increased, with resulting increase in light absorption, possibly by intramolecular hydration. The consistent preliminary decrease in absorption of light between 40° and 50° C., which is found in the fluid of *Phascolosoma*, does not obtain in horse serum (see DuNouy, 1945, The critical temperature of serum).

*Observations on the hemolytic action of sodium taurocholate.* MARIAN WILLIS, W. E. LOVE AND M. H. JACOBS.

The effects of hemolytic concentrations of sodium taurocholate on mammalian erythrocytes can be stopped short of hemolysis by suitable dilution with 0.3 M sucrose. Cells so treated can be kept with little change for hours. They show permeability to non-electrolytes that can be regulated within fairly wide limits by the concentration of taurocholate employed and to a lesser extent by the length of exposure. Too great an exposure may produce a permeability even to sucrose.

Taurocholate-treated cells give osmotic evidence in the following ways of an induced permeability to cations, the degree of which can also be varied by the character of the exposure: (1) immediate rapid shrinkage in pure sucrose solutions isosmotic with blood, (2) swelling and hemolysis in NaCl solutions of any strength, (3) a protective effect in NaCl solutions of low concentrations of sucrose, (4) shrinkage followed by recovery to more than the original volume on the addition of concentrated NaCl to cells in an appropriate mixture of NaCl and sucrose, and (5) a minimum rate of hemolysis of previously treated cells in NaCl solutions near the isoelectric point of hemoglobin.

While the swelling of cation-permeable cells is favored by increasing alkalinity above the isoelectric point of hemoglobin, as demanded by the Donnan principle, the production of a condition of cation-permeability by taurocholate falls off rapidly as the pH rises. The simultaneous existence of these two dissimilar types of action results in a somewhat complex overall pH effect. It also explains the observation of Ponder that according to the time of its addition alkali may either increase or decrease the rate of taurocholate hemolysis.

*Native protein networks and biological morphology.* DOROTHY WRINCH.

A major problem of biological morphology is the explanation of the relationship between the characteristic gross morphology or "habit" of an organism and its atomic structure. In mineralogy there is a comparable passage from the world of angstroms and the atomic pattern of a crystal to the visible level and crystal "habit." A possible approach to the biological problem may be suggested according to which the parallel use by biologists and mineralogists of the term *habit* is not just a coincidence, but the key to the problem.

According to the native protein theory of the structure of cytoplasm, (1) the essential entity is a network of protein units of low connectivity running throughout the organism, unicellular or multicellular, the plasma membrane of each cell being a localized "skin" of higher connectivity;

and (2) the interlinkings of the protein units, with or without non-protein units as intermediates, are of the degree of precision achieved by intermolecular associations in crystals (Wrinch, *Cold Spring Harbor Sym.*, 9: 218; 1941; *Collecting Net*, 17: 83; 1942). We now suggest that the non-protein material secreted, e.g., the successive siliceous shells of diatoms, and cellulose shells of desmids, the calcium carbonate shells of corals, are laid down on the plasma membranes with the same crystal-like precision, so that such structures may be viewed as intergrowths onto the underlying network of functional proteins, on which biological specificity depends, of essentially the same structural nature as the intergrowths of inorganic crystals (e.g. quartz onto calcite).

The "symmetry" of living organisms discussed by biologists in the branching of trees and corals, phyllotaxis, etc., is not symmetry in the classical sense: the actual point symmetry in each such case is a totally inadequate description of the morphological situation. Rather is it of the type encountered in such complex intergrowths as the dendritic forms, frequently encountered with gold, copper and other species, especially those belonging to the cubic system (*Dana's Mineralogy*, 1922; p. 173). As is well known, twins and other mineral intergrowths are, in many cases, direct indications of a major atomic theme of high symmetry (Wrinch, *Am. Mineral*, in press). Correspondingly a diatom shell is, like a snowflake, a significant complex of intergrowths, not a single crystal. Its biological essence resides in its relation to the underlying atomic patterns of the proteins in the plasma membrane and a cubic skeleton for protein molecules (Wrinch, *Biol. Bull.*, 89: 192; 1945) provides living matter with just such a major theme.

#### PAPERS READ BY TITLE

*Permeability of eggs of Arbacia punctulata to radioactive phosphorus.* PHILIP H. ABELSON.

Studies have been made of the permeability of unfertilized and fertilized eggs using radioactive phosphorus. Eggs in batches of 100,000, obtained by the conventional techniques, were exposed to filtered sea water containing  $5.8 \times 10^{-8}$  mg. phosphorus in the form of  $\text{Na}_2\text{HPO}_4$  per 100 cc. for varying periods of time. Aliquots of the eggs were washed four times under conditions which insured removal of all supernatant solution and freely diffusible phosphate. In the case of fertilized eggs, studies were made both at  $10^\circ \text{C}$ . and at  $23^\circ \text{C}$ . In addition the effect of a metabolic poison—4-6 dinitro-o-cresol—was observed. The generalized conclusion was that phosphorus take-up of the eggs was quite closely connected to cellular activity. Thus the amount of phosphorus taken up by the fertilized eggs was 40-fold greater than that of unfertilized eggs. It is interesting to note that oxygen metabolism has been found to differ by only a factor of three in this case. At  $10^\circ \text{C}$ . the amount of phosphorus found in the eggs was only one-seventh that found for a similar exposure at  $23^\circ \text{C}$ . As is well known the relative times for first cleavage are also about the same factor different. When  $1.6 \times 10^{-5}$  molar 4-6 dinitro-o-cresol is present oxygen metabolism is about doubled while cell division is almost stopped (Krahl, M. E., and G. H. A. Clowes, *Jour. Gen. Physiol.*, 23: 413; 1940). Under these circumstances phosphorus take-up was diminished by a factor of six. A survey of the fate of the radioactive phosphorus taken in at  $23^\circ \text{C}$ . showed that most went into the "acid soluble fraction" (phosphorus compounds soluble in 5 per cent tri-chloro acetic acid). With short exposures to radioactive phosphorus the "acid soluble fraction" contained ten times as much activity as the "acid insoluble fraction." Most of the "acid soluble fraction" was in the form of organic phosphate. One interesting feature was that the phosphorus uptake did not increase immediately after fertilization. A transition period of seven to ten minutes elapsed in which a new level of phosphorus intake was attained. One hour after fertilization the amount of traced phosphorus per million eggs ( $74 \mu$  diameter) was  $1.0 \times 10^{-8}$  mg. The rate of increase was  $2.0 \times 10^{-8}$  mg. per million per hour. This rate remained constant during the next four hours.

*Photosynthesis of Ulva.* MARY BELLE ALLEN.

The green marine alga *Ulva lactuca* has been found to possess properties which make it a highly desirable organism for studies of photosynthesis. Thallae can easily be washed free of



possible bacterial contamination and cut into small pieces for manometric measurements. The maximum rate of photosynthesis in 0.1 M  $\text{KHCO}_3$  in sea water is 2.5 mm.<sup>3</sup> oxygen/min./ $\gamma$  nitrogen, as compared with 0.03 mm.<sup>3</sup> oxygen/min./ $\gamma$  nitrogen under similar conditions for the algae commonly used in physiological studies, *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. The respiration of freshly collected samples is less than one per cent of photosynthesis, while in *Chlorella* and *Scenedesmus* respiration is 5–10 per cent of photosynthesis. When *Ulva* is kept in the laboratory for several days, even in running sea water, the rate of photosynthesis drops and respiration increases. Fresh samples are extremely resistant to cyanide, photosynthesis being unaffected by 0.001 M KCN and only 60–75 per cent inhibited by 0.01 M KCN. Old samples, with lower rates of photosynthesis, are more cyanide sensitive.

When a sample of *Ulva* which has been illuminated is placed in the dark, evolution of oxygen and uptake of carbon dioxide continue. The rate of oxygen evolution and carbon dioxide absorption slowly falls off in the dark, the time required for decay depending on the rate of photosynthesis. Two hours is required for the gas exchange of a fresh sample to fall to zero in the dark, while with old samples the effect is over in 10–15 minutes. The photosynthetic quotient measured by the Warburg indirect method is  $1.05 \pm 0.06$  during illumination and  $0.98 \pm 0.03$  during the period of oxygen evolution and carbon dioxide uptake in the dark following illumination. The intermediates of photosynthesis in *Ulva* must therefore be much longer lived than those in most plants, perhaps because they are not as rapidly removed by respiratory reactions.

#### *Chromoproteins in the tentacles of Physalia.* ERIC G. BALL AND OCTAVIA COOPER.

In a study initiated to learn the nature of the toxic substance present in the stinging tentacles of *Physalia* several chromoproteins have been separated. The ground tentacles are extracted with water, centrifuged, and to the turbid bluish black supernatant ammonium sulfate is added to 35 per cent saturation. A heavy purplish-brown precipitate is centrifuged off. Further fractionation of this precipitate or characterization of its color has proved difficult. The bluish-green supernatant is brought to 60 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  and centrifuged. The colored precipitate is dissolved in water and dialyzed until salt free. A light blue precipitate forms, leaving a clear yellowish-green supernatant.

The blue precipitate can be dissolved in 0.9 per cent NaCl solution to give a slightly turbid solution with an absorption peak at 580–600 m $\mu$ . Acid ethyl alcohol added to the blue precipitate denatures the protein and yields a clear green solution with absorption maxima at  $\lambda$  700 m $\mu$  and 370 m $\mu$ . Treatment of the blue protein precipitate with neutral ethyl alcohol denatures the protein but extracts very little color. Extraction of the precipitate now with 0.1 M phosphate buffer, pH 6.8, yields a clear blue solution of the prosthetic group with a well defined absorption peak at  $\lambda$  585 m $\mu$ . This suggests that the blue form of the prosthetic group exists as a salt and that its combination with protein may involve a salt linkage. Tests for Cu and Ni gave negative results.

The greenish-yellow solution obtained after dialysis shows an absorption peak at  $\lambda$  450 m $\mu$ . All attempts to obtain its prosthetic group in solution free of its protein carrier lead to complete destruction of the color.

Solutions of both of these colored proteins exhibit lethal properties upon injection into *Fundulus*. Heat destroys these toxic properties. However, proof that the toxic action of the tentacles is due to these chromoproteins must await further work.

#### *Effects of hydrogen ion concentration and of certain buffer systems upon the luminescent reaction of Cypridina luciferin and luciferase.* AURIN M. CHASE.

Luciferin, extracted from the ostracod crustacean, *Cypridina hilgendorffii*, was carried through two cycles of purification by Anderson's procedure (*Jour. Gen. Physiol.*, 19: 301; 1935). Luciferase, from *Cypridina*, was partially purified by dialysis.

The luminescent reaction, measured by Anderson's method (*Jour. Cell. Comp. Physiol.*, 3: 45; 1933), was studied from pH 5.5 to 8.7. Three buffer systems were used:  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$ , and  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$ ; all 0.067 M for phosphate and 0.01 M for sodium chloride. Attempts to extend the pH range with borate and barbiturate buffers

were abandoned because the borate radically altered the luminescent reaction and the barbiturate abolished it.

Two distinct effects of pH were found, one upon the total light of the reaction and the other upon the first order reaction velocity constant.

Total light decreased with increasing pH, to a value at pH 8.7 about 20 per cent of that at pH 5.5. This effect was not due to non-luminescent oxidation of part of the luciferin during the reaction as in the case of temperature (Chase and Lorenz, *Jour. Cell. Comp. Physiol.*, 25: 53; 1945), but apparently to a specific effect of pH upon the light-emitting system analogous to salt effects found by Anderson (*Jour. Amer. Chem. Soc.*, 59: 2115; 1937).

The velocity constant of the reaction was maximal at about pH 7.2. It decreased to about 50 per cent of the maximal value at pH 8.7 and to about 15 per cent of the maximal value at pH 5.5.

At any given pH the velocity constant of the reaction (but *not* the total light) was affected by the sodium:potassium ratio of the phosphate buffer. For example, at pH 6.8 the velocity constant was measurably greater with the  $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$  buffer or with the  $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$  buffer than with the  $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ . This would seem to indicate some sort of sodium-potassium antagonism in this enzyme system.

#### *Retardation of Arbacia egg cleavage by dinoflagellate-contaminated sea water (red tide).* IVOR CORNMAN.

Two specimens of "red tide" were generously supplied by Dr. P. S. Galtsoff, Director of the U. S. Fisheries Laboratory, Woods Hole, Massachusetts.

An untreated decomposing sample of sea water taken from an area stained red by *Gymnodinium* when diluted 1:10 retarded *Arbacia* egg cleavage by one hour—100 per cent increase in cleavage time—if added 10 minutes after fertilization. When most of the  $\text{H}_2\text{S}$  was pulled off with a vacuum pump, the delay at 1:10 was only 3 minutes and at 1:5 was 15 minutes. Four days later all odor of  $\text{H}_2\text{S}$  was gone, but retardation was essentially the same, 9 per cent at 1:10 and 27 per cent at 1:5. Cytolysis resulted from exposure to 1:2. This inhibitory potency is equal to that of crude filtrate from some *Penicillium* cultures. This sea water sample killed *Fundulus* in 2 hours at 1:2 and in 5½ hours at 1:10. A "red tide" plankton sample suspended in sea water and preserved with  $\text{CHCl}_3$  was evacuated until no odor of  $\text{CHCl}_3$  remained. At 1:10 this retarded cleavage 10 minutes. *Fundulus* in this sample diluted with an equal part of sea water lost equilibrium and became sluggish in 2 hours, and in 6 hours at 1:10. They recovered motility in fresh sea water, but subsequently died. There appears to be some parallel between the toxicity to fish and to dividing eggs, but whether the same poison acts upon both and whether decomposition plays an important role remain to be determined. Studies conducted near the site with fresh samples of sea water and dinoflagellates should prove more helpful if uncontaminated test organisms are available.

#### *Abnormalities of mitosis in tail-tips of Triturus torosus.* DONALD P. COSTELLO AND CATHERINE HENLEY.<sup>1</sup>

The material of this study consisted of 485 larvae developing from a batch of eggs of *Triturus torosus* shipped by express from Stanford University, California to Chapel Hill, North Carolina, in the usual type of thermos jug used for embryological materials. These larvae were raised under normal laboratory conditions. At the appropriate stage, the tails were clipped and prepared as whole mounts for cytological examination. Three additional successive clippings of the regenerated tails of the surviving larvae were performed at approximately 12-day intervals, during the period of daily feedings following yolk resorption, giving a total of 1,252 tail-tip preparations. The first regenerates are the best preparations. These (and others) show an unusually high percentage of cytological abnormalities of the types described by Alberti-Poltzer (1923, 1924) after X-ray treatment of the corneal epithelium of *Salamandra maculosa*. The resting nuclei are of various sizes and forms, making it impossible to use nuclear size as a criterion of heteroploidy. Fragmentation of resting nuclei, and nuclear necrosis, with the production of numerous small basophilic areas, are among the causes of variation

<sup>1</sup> Aided by a grant from the Carnegie Research Fund of the University of North Carolina.

in nuclear size. Numerous mitotic abnormalities were also observed. These include: multi-polar spindles; spindles from which chromosomes have been lost; lagging chromosomes and lagging or lost chromosome fragments at anaphase; bridges between anaphase or telophase chromosome groups, or between daughter nuclei; basophilic granules (chromatin?) outside spindle, etc. The chromosomal condition of these tail-tips includes euploidy and aneuploidy, a remarkable number showing mosaic areas. A preliminary survey of these mosaics reveals at least the following types: haplo-diploid; hypodiplo-diploid; hyperdiplo-diploid; diplo-triploid; diplo-tetraploid; diplo-pentaploid; diplo-hexaploid; diplo-octoploid.

Before attributing these cytological anomalies to any specific cause it is necessary to make a survey of natural heteroploidy in material collected and fixed in California.

### *A new form of the hydroid Stylactis.* SEARS CROWELL.

*Stylactis arge* Clarke and *S. hooperi* Sigerfoos have not been critically compared so there is doubt as to their specific distinctness (for references see C. M. Frazer, Hydroids of the Atlantic Coast of North America). In late July this year Mr. Milton Gray collected and brought me a specimen of *Stylactis*, found growing on a *Mytilus* shell at Woods Hole. I have compared it with specimens on shells of *Nassa* (*Ilyanassa*) *obsoleta*.

In the following respects it differs from the typical *S. hooperi* as observed on *Nassa*: (1) Spines are absent. (2) The tentacles may be visualized as "... arranged in two verticils ... those of the lower circlet sometimes shorter than those of the upper one," as described for *S. arge* by Clarke. (3) Gonophores are not directly below the tentacles but about one-third the distance from tentacles to base, as indicated in Clarke's figures of *S. arge*. (4) Tentaculo-zoids, not hitherto reported in the genus, are scattered among the other hydranths. These zoids are not enlarged terminally, not capable of forming a spiral, and not so long, when extended, as the largest gastrozoids.

The specimen differs from *S. arge* of Clarke in having shorter hydranths than his illustrations indicate, and more tentacles than he states are present. However, his description is equivocal on both points.

The specimen may be interpreted in one of three ways: (1) It may be *S. arge*. (2) It may be a new species. (3) It may illustrate the variation in a single species, as is likely true of *Stylactis arge* and *S. hooperi*.

### *The effect of X-rays on chromosomes and nucleoli in ovarian eggs of the salamander, Triturus pyrrhogaster.* WILLIAM R. DURYEE.

The giant chromosomes of *Triturus pyrrhogaster* ovarian eggs react to X-irradiation in the same way as previously determined for those of the frog.

Four criteria of X-ray damage have been found. These are: (a) fragmentation of the chromonemata, (b) progressive disintegration of the lateral chromomere loops, (c) colloidal changes in nucleoli, and (d) gel  $\rightarrow$  sol transformations in the central nucleoplasmic chromosome frame. High intensity radiation at the approximate rate of 6,120 r/min. was employed in dosages graded from 10,000 to 70,000 r, with the majority of the experiments at the 60,000 r level. Whole eggs were exposed in ordinary Amphibian Ringer buffered to pH 7.6, while isolated nuclei and nuclear-free brei were irradiated in Ca-free N-medium (NaCl 0.66 g., KCl 0.014 g., dist. H<sub>2</sub>O 100 cc.).

Multiple fragmentation of chromosomes appeared immediately following as little as 30,000 r. With 60,000 r or more 30-50 chromosome fragments could be found in each nucleus. The average fragment was of the order of 45-50  $m\mu$  in length, as compared with normal chromosomes 180-700  $m\mu$  long. Within these dosage limits, degree of fragmentation was roughly proportional to the amount of radiation. Some changes in lateral loops could be detected with as little as 10,000 r. By 30,000 r nearly all lateral loops had been stripped from the chromonemata, leaving the 13 chromosome pairs appearing as granular cylinders. Following exposures to greater dosages (30,000 to 60,000 r) large numbers of loop fragments were observed throughout the nucleoplasm surrounding the chromosomes. Above 60,000 r nearly all loop fragments had dissolved into numerous granules or micronucleoli (0.5-2.0  $m\mu$  diameter).

Nucleolar changes were strikingly evident. While normal Stage 4 nucleoli were clear

polygonal or irregularly oval bodies, irradiated nucleoli had large vacuoles and granular interiors. Irradiation increased the normal average size from 15-20  $m\mu$  diameters to 30-45  $m\mu$ . Following the higher dosages clumping of nucleoli into masses over 150  $m\mu$  in diameter was frequent.

Changes in colloidal condition of chromosome frame substance were recognized by sinking and displacement of chromosome pairs. Solvation of the supporting gel usually required at least 50,000 r.

Eight sets of isolated nuclei were irradiated directly with 60,000 r. One set was given 100,000 r. In contrast to effects obtained with whole cells, all chromosomes were found normal in length and no evidence of fragmentation could be detected. Even with the highest dosage lateral loops were only partially dissolved. Nucleoli occasionally appeared vacuolated, clumped and with surface markings. Solvation of chromosome frame substance occurred in 80 per cent of directly irradiated nuclei.

Four other permutations of these conditions were examined: (a) non-irradiated nuclei exposed to normal cell brei, (b) non-irradiated nuclei exposed to X-rayed cell brei, (c) exposure of normal eggs to irradiated Ringer solution, and (d) non-irradiated nuclei exposed to alkalinized normal Amphibian Ringer, pH 8.0. In (a) no marked changes occurred until after 12 hours when slight vacuolization of nucleoli and some lateral loop damage was found. Irradiated brei, on the contrary, in less than 1 hour dissolved the lateral loops, vacuolated the nucleoli and displaced the chromosomes, but did not cause fragmentation. Irradiated Ringer produced no significant changes in whole cells. However alkalinized Ringer alone hydrolyzed lateral loops, caused nucleolar vacuoles and solvated the chromosome frame.

It is concluded that high dosages of X-irradiation will cause immediate fragmentation of egg chromosomes, loss of lateral chromomere loops and vacuolization of nucleoli when the nucleus is in the cell. On the other hand isolated nuclei and chromosomes do not react markedly when irradiated directly. Intermediate degrees of nuclear damage between the above extremes may be produced by exposure of non-irradiated nuclei to X-rayed cytoplasm or simply to alkalinized Ringer. These data are consistent with a hypothesis that X-ray damage to nuclear components is not primarily a direct effect, but an indirect one, probably caused by chemical changes in the cytoplasm.

### *The chromosome number of Triturus torosus.* CATHERINE HENLEY AND DONALD P. COSTELLO.<sup>1</sup>

A total of 1,497 permanent whole-mount tail-tip preparations were made of the original and regenerating tails of 763 control and 14 experimental (cold-treated) *Triturus torosus* larvae. These included 244 larvae fixed in California and 533 shipped by express to Chapel Hill. A preliminary survey of the mitotic figures on these preparations has been made, and 126 metaphase chromosome groups studied and drawn. Many of these figures are from larvae with apparently normal diploid tail-tips throughout (criteria: nuclear size and normal mitotic figures), others from tail-tips showing obvious mitotic abnormalities (see abstract by Costello and Henley).

Since the chromosome number of this species has not been recorded, it is of interest to present the data on the 126 chromosome counts made to date. Six metaphases of four individuals which were obviously haploid showed 11 chromosomes. The remaining individuals included larvae which were diploid, triploid and mosaic heteroploids. Sixty-four of the metaphase figures from these had 22 chromosomes; eleven, 21 chromosomes; nine, 23 chromosomes; ten, 24 chromosomes. In five triploid figures, 30 or 31 chromosomes could be counted. In tetraploid and pentaploid figures, 42 and 51 chromosomes, respectively, were seen. The remaining counts varied from 15 to 27 chromosomes.

In crowded figures, chromosome counts are likely to be too low rather than too high. Since we frequently observed indications of chromosome elimination and lagging at anaphase in the abnormal tail-tips, it is likely that many of the figures examined were hypo- or hyperdiploid. In general, the evidence is in accordance with the conclusion that the diploid chromosome number of *Triturus torosus* is 22.

<sup>1</sup> Aided by grants-in-aid from the Carnegie and Smith Research Funds of the University of North Carolina.

*Polyphyodonty in the dogfish, Mustelus canis.* JOHN D. IFFT AND DONALD J. ZINN.

The commonly accepted theory of tooth replacement in sharks describes them as moving forward from behind to replace those lost in front. This theory has been denied in recent years by Cawston. Since no direct observations of replacement apparently exist it was thought to be of some interest to determine experimentally the manner of replacement.

In one group of smooth dogfish two rows of teeth in the center section were extracted. All of the teeth back of the extracted area and all of the lateral teeth, except for the remaining ones of the first two rows, were marked with silver nitrate. In twenty-three days in some of the animals all of the teeth were replaced; in others only one row was replaced. The replaced teeth, as well as the rows lateral to them, had silver nitrate markings indicating that all of the teeth had rolled forward. Sections show that tooth buds are produced only in back of the entire erupted tooth area and not in any other location.

In another group the tooth-bud area was cauterized and as a result tooth replacement was inhibited. In others, triangular areas of teeth were extracted and replacement occurred without disruption of the normal arrangement of the teeth. All of the evidence seems to indicate that the original polyphyodont theory of tooth replacement is correct. The rate of replacement is apparently variable.

*A method to immobilize fish for cardiac and other experiments with procaine.*  
BRUNO KISCH.

The well known difficulty of performing cardiac experiments on fish was overcome with the following method of a preparation of the immobilized animal, practical without any loss of blood.

The animal is placed on its stomach. With a syringe and a 20 gauge medical needle for intravenous injections the skull is pierced somewhat caudally of the middle of a line connecting both eyes. According to the size of the fish  $\frac{1}{4}$  up to 1 cc. 5 per cent solution of Novocain (Procaine hydrochloride) is injected intracranially and the needle removed.

The fish is immobilized and apparently also anesthetized satisfactorily within a few minutes and the effect lasts from  $\frac{1}{2}$  to 1 hour. A 1 per cent solution has no satisfying result but produces especially in dogfish with surety a long lasting nystagmus. A 10 per cent solution is dangerous because it more often kills the animal. Between 3 and 7 per cent is the most favorable concentration. The animal continues to breathe and if the head is submerged in a small seawater tank the heart can be used for a long time for experiments. If the fish is kept without water the heart beats also for a long time well. Intracranial injection of the same amount of saline or hypertonic saline solution has no similar effect.

The usual fenestration of the chest has to avoid lesions of the vessels to avoid loss of blood.

If the animal narcotized in this way (especially dogfish) is not operated on but brought back into the seawater tank it shows long lasting undulatory movements of its body, does not react to stimuli but recovers after about 1 hour completely. Whether the procaine during this time is reabsorbed or destroyed by a procaine esterase could not yet be ascertained.

This method will be useful for other experiments too; for instance, for metabolic investigations on the muscle or electric organ.

*Cardiographic studies on the heart of fish.* BRUNO KISCH.

Different species of fish (Selachiens and Teleostei) were investigated electrographically with so-called unipolar leads, the exploring electrode being placed on the surface of the heart or inside of auricle or ventricle. In spite of fish having only one auricle and one ventricle their cardiograms are of the same type as the ones of mammals and man. The asynchronism of excitation of the different parts of the ventricle makes for the complicated form of the fish electrocardiogram. It could be proven that the part of the ventricle first activated is its upper dorsal plane and mostly the left side of the base. Always the base of the ventricle is earlier activated than the apex. The endocardial surface is not activated earlier than the earliest activated parts of the surface of the ventricle but synchronously, in some instances even later.

The type of cardiograms registered from the inside of the ventricle depends to a high degree on the part where the electrode is placed. In Selachians there is a clear differentiation between the endocardial electrogram of the right and of the left inside of the ventricle. The endocardial electrogram depends, of course, also on the type of contraction of the ventricle (partial systoles, normal systoles, extrasystoles, fractionated systoles).

The electrogram of the auricle is normally polyphasic showing a quick and a slow group of deflections. Sometimes it is bifid due to the asynchronous contraction of the different parts of the big auricle and its different appendages.

It was proven for the first time that the muscular bulbus cordis of Selachians shows not only contractibility but also a high degree of automatism since in the exhausted heart of *Raja erinacca*, for instance, the bulbus is sometimes the pacemaker of the heart beat. This fact was also registered by means of electrocardiography.

*Alkaline phosphatase activity in oöcytes of various marine invertebrates.* E. J. KRUGELIS.

Using the cytochemical technique of Gomori, alkaline phosphatase activity was detected in the oöcytes of several marine invertebrates, including *Arbacia*, *Asterias*, *Cynthia*, *Chaetopterus*, and *Mactra*. This activity is present when sodium glycerophosphate, adenylic acid, or yeast nucleic acid are used as substrates for the enzyme. The cytoplasm of the oöcytes contain very little detectable enzyme activity while the germinal vesicle including nuclear sap and nucleoli shows a definite reaction in each form investigated. Basophilia is found in the cytoplasm and the nucleoli, while the germinal vesicle is faintly basophilic. Thus cytoplasmic basophilia and phosphatase activity seem independent in location in the oöcyte stages. Both basophilia and phosphatase activity occur together in all nucleoli except those of *Mactra*. This form possesses an amphinucleolus which includes a small basophilic nucleolus and a large oxyphilic nucleolus. Only the large oxyphilic nucleolus contains the enzyme activity. Thus, in the case of *Mactra* at least, basophilia seems completely separate from the location of enzyme activity.

*Alkaline phosphatase activity in developmental stages of Arbacia.* E. J. KRUGELIS.

The occurrence of alkaline phosphatase activity in *Arbacia* has been detected by use of the Gomori cytochemical technique. In the oöcytes, enzyme activity was detected only in the germinal vesicle. During this stage the vesicle stains with acid dyes such as fast green, but stains very faintly with the basic dye, toluidine blue or with Feulgen. The cytoplasm of the oöcyte, however, is strongly basophilic with toluidine blue. This basophilia of the cytoplasm is interpreted on the basis of Brachet's observations (*Embryologie Chimique*, Liège, 1945) as ribose nucleic acid. In the unfertilized mature egg, the fertilized egg, and early cleavage stages, the cytoplasm is the site of both basophilia and enzyme activity. Only sodium glycerophosphate was used as the enzyme substrate for these later stages. During blastula, gastrula, and plutei stages the cytoplasmic enzyme reaction seems to decrease parallel to the decrease in cytoplasmic basophilia. The nuclear enzyme activity increases in these stages, parallel to an increase in nuclear basophilia and Feulgen reaction.

This suggests that the enzyme and ribose nucleic acid have independent locations during oögenesis. During maturation the enzyme may be liberated into the cytoplasm and then parallels ribose nucleic acid content in the cytoplasm. This parallel location of the enzyme activity and nucleic acid is similar to the condition found in the late cleavage stages of amphibian embryo and suggests a possible active association during development.

*A comparative study of iron content in marine invertebrates.* SISTER ELIZABETH SETON MACDONALD AND CHARLES G. WILBER.

It is well known that sea water ordinarily contains only minute traces of iron in solution (see Cooper, *Proc. Roy. Soc. London (B)*, 118: 419; 1935). Many invertebrates have great power of concentrating iron in the tissues, but there is a lack of quantitative information concerning the amount of iron in the body fluids of various marine invertebrates. A series of

colorimetric analyses were, consequently made to ascertain the iron content of the body fluids in several invertebrate animals. The amounts of iron (mean value of 10 animals unless otherwise indicated by number in parentheses after the species name) in mg./100 cc. fluid are as follows: *Phascolosoma gouldii*, 21.1; *Amphitrite ornata*, 9.8; *Glycera americana* (8), 15.5; *Nereis pelagica* (6), 9.6; *Chaetopterus variopedatus*, 3.1; *Venus mercenaria* (5), 2.0; *Asterias forbesi* (2), 2.8. The amount of iron is much higher in the annelids studied than in the mollusc or echinoderm. Such would be expected in view of the presence of heme pigments in the fluids of the former but not of the latter. It is interesting to observe, however, that there are consistently measurable amounts of iron in the mollusc and starfish, indicating that iron is in some way necessary for the general metabolism of these forms as well as for the annelids. A study of the variation in iron content in the above animals throughout the year is now in progress.

*Fertilization studies on a non-conjugating race of Paramecium aurelia.* CHARLES B. METZ AND MARY T. FOLEY.<sup>1</sup>

Metz (*Jour. Exp. Zool.*, 105: 115; 1947) has shown that mixture of formalin-killed and living *Paramecium aurelia* of opposite mating types results in (1) specific clumping between living and dead animals, (2) induction of pseudo selfing and (3) activation (macronuclear breakdown and presumably other conjugation effects) of the living animals. In an attempt to discover the initiating mechanisms of activation and pseudo selfing, much of the previous work has been repeated on an abnormal (CM) race obtained through the kindness of T. M. Sonneborn. These CM animals give normal mating reactions but fail to conjugate.

It is found that formalin-killed CM animals induce pseudo selfing in living normal animals. However, living CM animals failed to self when treated with formalin-killed normal animals although the CM animals clumped strongly and specifically with these dead animals. From these results it seems highly unlikely that pseudo selfing involves transfer of mating substance from dead to living animals (as previously suggested). It is now believed that pseudo selfing depends upon interaction of specific "holdfast" substances which appear when the animal is activated but which are not mating type specific.

Formalin-killed CM animals can also induce macronuclear breakdown in single isolated living normal animals. From this it follows that the CM animals possess the activation initiating mechanism. However, living CM animals cannot be activated by treatment with dead normal animals. Therefore a block must prevent activation from proceeding much beyond the initial step in the CM animals. This evidence strongly favors the view that interaction of mating type substances initiates activation in *P. aurelia*.

Since the CM animals undergo natural autogamy, it follows that natural autogamy is not initiated through the same mechanism that operates in conjugation. An analogous situation may obtain in parthenogenesis, namely that the activation initiating mechanism of normal fertilization is by-passed in parthenogenesis.

*Thecal morphology of some dinoflagellates of Woods Hole, with special reference to the "ventral area."* DASHU NIE.

A total of eighteen species belonging to five genera was subjected to this study. *Exuviaella almonda* sp. nov., *E. marina*, *Prorocentrum schilleri* and *P. micans*, which represent all the Prorocentridae examined in this study, were revealed to have the same architecture. They have besides the two prominent bivalve-like plates four small platelets, lodging in the concavity of the left plate at the anterior end. Of these, three form the flagellar pore or pores, and one forms the spinose platelet. The number and disposition of these platelets are reported here for the first time.

The entity of the "ventral area" or sulcus of the genus *Diplopsalis* as well as the genus *Peridinium* has been found to consist invariably of six plates, namely, left and right anterior plates, left and right flagellar pore plates, the connecting plate and the posterior plate. These plates have been isolated in the following species: *Diplopsalis lenticula* f. *minor*, *D. asym-*

<sup>1</sup> Aided by a grant from the National Institute of Health, U. S. Public Health Service.

*metrica*, *Peridinium pallidum*, *P. globulus* var. *quarnerense*, *P. mariclebourae*, *P. conicum*, *P. claudicans*, *P. oceanicum*, *P. leonis* and *P. depressum*.

Four species of the genus *Goniaulax*, namely, *G. diegensis*, *G. digitale*, *G. spinifera* and *G. monocantha*, have been examined, and the result turned out to be quite different from that of Kofoid's (1911). A new plate formula is suggested: 3', 3a, 6'', 6, 5''', 2''''', + 6 v.a.p. The reason for such a change is discussed.

*Plastid development in the scutellum of Triticum sativum and Secale cereale.*

JOHN A. O'BRIEN, JR.

During germination of grains of *Triticum* and *Secale*, there are present in cells of the epithelium and the parenchyma of the scutellum, characteristic solitary aggregations of cytoplasmic elements which are preserved by mitochondrial fixatives. Each element at one stage is composed of a swollen head portion and an attenuated tail. They are so assembled that the heads are directed to the periphery and the tails toward the center of the group. The aggregation occurs in a differentiated region of the cytoplasm which, when preserved by certain fixatives, appears optically homogeneous and is thus set off from the granular cytoplasm. Usually the aggregation lies immediately adjacent to the nucleus. The elements comprising the aggregation eventually become dispersed in the granular cytoplasm and can be traced through several stages of dispersion. As the aggregation first begins to break up, the tail portion of the individual element develops a small knob at its distal end and the head portion diminishes slightly in size. After dispersion, the elements assume the characteristic form of plastids in fixed cells and vesicles which develop in them can be demonstrated to be starch grains. In some groups, the formation of starch grains occurs even before dispersion.

W. J. V. OSTERHOUT. No abstract submitted.

*Vitamin A and lipid metabolism in the frog.* CHARLES G. WILBER AND MARY E. F. TWOMEY.

Very little is known concerning the effect of vitamins on lipid metabolism in poikilothermal vertebrates. Consequently an investigation was undertaken to ascertain the effect of vitamin A on lipid metabolism in the frog. Frogs were fed corn oil in capsules and corn oil plus vitamin A in capsules respectively. After 48 hours the livers and kidneys were removed and analyzed for phospholipid, cholesterol, and fatty acid. The results were compared with those obtained from the analysis of unfed frogs used as controls. It was found that there was an increase of cholesterol, fatty acid, and total lipid in the liver and kidney of frogs fed corn oil alone but that there was a decrease in phospholipid in the same organs. Frogs fed fat plus vitamin A, on the other hand, showed an increase in phospholipid and a decrease in other lipids in the kidney and liver. The results clearly indicate that a high fat diet in the frog brings about a marked increase in the cholesterol and fatty acid in both liver and kidney. It is, moreover, evident that vitamin A stimulates to a marked degree the use of fat in the frog. These results are in accord with those obtained by other investigators using homoiothermal vertebrates (see Monaghan, *J. Biol. Chem.*, 98: 21; 1932; Asada, *Biochem. Zeitschr.*, 142: 44; 1923 and 144: 203; 1924). The present investigation indicates that in the frog diet vitamin A is an essential component. On a diet lacking the vitamin there is an accumulation of lipids in the liver and a decrease in the synthesis of phospholipid by that organ.

*Alkaline phosphatase in the early development of Fundulus heteroclitus.* ANITA ZORZOLI.

A study of the localization of alkaline phosphatase in the development of *Fundulus heteroclitus* from late cleavage to the post hatching period when the yolk has been completely absorbed was carried out using the histochemical technique of Gomori. Each specimen was sectioned serially at 6  $\mu$ , cut into 2 section pieces and mounted on separate slides so that adjacent sections could be used for routine staining and for controls. Incubation was carried on



for 12 and 24 hours at room temperature (approx. 26° C.) using Na  $\beta$ -glycerophosphate as substrate for the reaction.

There is a conspicuous lack of phosphatase activity in the large cells of the late cleavage and blastula stages as well as in the smaller cells of the gastrula stages. The first positive reactions occur at the beginning of differentiation of the nervous system. At this stage and shortly after, the nuclei of the neural cells stain deep brown-black. The cytoplasm is also positive but less strongly so. Outer ectoderm, endoderm and mesoderm cells stain uniformly positive and slightly less intensely than the nervous system. The large notochordal cells show the weakest reaction.

As tissue and organ differentiation proceeds, new regions showing strong activity appear while others decrease in activity. For example, by the time the cerebral hemispheres have formed there is a difference in intensity of reaction within the central nervous system itself, the anterior regions being more strongly positive than the posterior regions. Within the spinal cord there is a distinctly stronger reaction in both nuclei and cytoplasm of the dorsal third as compared with the more ventral regions.

Increase in phosphatase activity with increase in differentiation is shown in the gut. Both nuclei and cytoplasm stain more deeply at the time of hatching as compared with earlier stages and in addition there is developed an intense blackening at the proximal surface of the mucosal cells. This localization is reminiscent of the type seen in the small intestine of many mammalian forms.

PROGRAM AND ABSTRACTS OF SEMINAR PAPERS PRESENTED AT  
THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1947

JULY 15

*Effects of sodium azide on the rate of amphibian development.* THOMAS S. HALL  
AND FLORENCE MOOG.

In the course of an investigation of the effects of sodium azide upon the developing egg of the grass frog, *R. pipiens*, it was observed that exposure to this substance can retard or suppress development for considerable periods without preventing the possibility of normal development after termination of treatment. This response is being studied experimentally with a view to determining (a) the exact conditions under which it may be expected (results reported here) and (b) physiological factors involved (experiments in progress). The first phase of this investigation has brought to light three principal facts. (1) Under exposure to solutions of sodium azide (in 10 per cent amphibian Ringer's solution at *ca.* pH 7.2), varying in concentration from  $7 \times 10^{-6}$  to  $5 \times 10^{-5}$  M, early stages (cleavage to late neurula) undergo reduction in the rate of their development. It proved possible to continue exposure for 2 to 4 days, depending upon the concentration, without precluding the possibility of normal progress after removal from azide solution. Such treatment reduced the rate to less than half of normal. (2) Even where treatments were sufficiently severe (solutions stronger than  $10^{-4}$  M) to induce complete arrest shortly after the treatment began, further development could occur. Individuals held at the gastrula stage for 48 hours went on to develop with every appearance of normality after treatment was stopped. In special instances, development was suspended for periods up to 86 hours; in these cases, however, not all individuals so treated developed normally, the incidence of abnormality increasing with the duration of the exposure. (3) If the organism was first exposed to solutions strong enough to cause complete arrest (e.g.,  $2 \times 10^{-3}$  to  $5 \times 10^{-3}$  M, for 3 to 12 hours), it was found to be subsequently more sensitive to weak azide solutions than were previously untreated individuals. The mechanisms responsible for the foregoing results are under investigation.

*Nucleic acids as growth factors in Drosophila.* CLAUDE A. VILLEE AND HAROLD  
B. BISSELL.

*Drosophila* larvae of wild, vestigial, Bar, Double Bar, dachsous, four-jointed, aristapedia and tetraltera stocks were grown on sterile, chemically defined media containing salts, agar, amino acids, cholesterol, lecithin, ribose nucleic acid and sucrose in which ribonucleic acid was replaced by nucleotides, or purines and pyrimidines, singly or in combination. Larvae grew as well on nucleotides as on ribonucleic acid; the growth-promoting effect of ribonucleic acid resides not in RNA as such but in the purine and pyrimidine constituents of RNA, and especially in adenine. Guanine seems to have some function in regulating the growth stimulating action of adenine. Desoxyribonucleic acid inhibited growth and pupation of the larvae. The vestigial stock, in contrast to fly and Double Bar, was able to survive in the presence of benzimidazole, a structural analogue of the purines, and seems to have some biochemical mutant which enables it to utilize benzimidazole in place of the purines. One phenocopy was observed in the course of the experiments: a Double Bar larva grown on a medium lacking sucrose and nucleic acid developed into a fly whose eyes were large and oval-shaped, approaching the wild phenotype. The flies hatching out of larvae grown on experimental media were fertile and in many tubes a second generation of flies was raised.

ANNA R. WHITING. No abstract submitted.

JULY 22

*The effects of podophyllin on the maturation and cleavage of the starfish egg.*

IVOR CORNMAN.

In a podophyllin concentration of 100 mg./L, breakdown of the germinal vesicle, penetration by the sperm, lifting of the fertilization membrane, and completion of cleavage by eggs in anaphase are to all appearances normal. On the contrary, the meiotic or mitotic spindle is quickly inactivated at 1 mg./L. The chromosomes released by spindle destruction form karyomeres which can engage in pseudomitotic cycles. If dosage is begun in the germinal vesicle stage, the released chromosomes or their karyomeres are unable to migrate to the periphery, but show evidence of responding to the meiotic rhythm by going through at least one cycle involving dissolution, chromosome formation, and reformation of the vesicular karyomeres. If the egg is fertilized or artificially activated, it goes through the same cycle. The karyomeres grow and coalesce, filling about half the volume of the egg by 24 hours. Occasionally they fuse to form a single nucleus which at 11 hours is  $\frac{5}{8}$  the diameter of the germinal vesicle and contains 1 to 3 nucleoli. Up to 30 karyomeres have been counted ( $2n=36$ ), and up to 18 in eggs blocked just after completion of the second polar body. Variation in size permits the interpretation that some karyomeres contain more than one chromosome. The karyomeres persist as discrete bodies following disintegration of the egg, showing that they possess membranes and are not mere differentiated areas of cytoplasm. In 12 hours (or sooner if the eggs have been washed free of podophyllin or if they have been fertilized) there is irregular division showing no relation to the distribution of karyomeres. In the living eggs there sometimes appear to be asters participating in this division, but sections have not yet been studied to determine if true cleavage processes or merely pathological fragmentation are involved. Threshold doses (ca. 0.2 mg./L) which inhibit the early cleavages permit proliferation of the asters and eventually simultaneous division of the eggs into 16-32 cells, which subsequently divide to produce a normal blastula.

*Preparation of a soluble cytochrome oxidase.* W. WAINIO, S. COOPERSTEIN, S. KOLLEN, AND B. EICHEL.

Cytochrome oxidase occupies a key position in the chain of enzyme reactions that catalyzes the dehydrogenation of substrates, because the oxidase apparently is the enzyme that "activates" molecular oxygen. Much work has been done in an attempt to free the enzyme from the complex of which it is a part in order that it might be more fully characterized. All attempts, with the possible exception of a partially successful attempt by Haas (*Jour. Biol. Chem.*, 148: 481; 1943), have failed to yield a soluble preparation.

The work of Hopkins, Lutwak-Mann, and Morgan (*Nature*, 143: 556; 1939) and Keilin and Hartree (*Proc. Roy. Soc. London, B*, 127: 167; 1939) suggested to us the use of bile salts to dissolve the cytochrome oxidase complex. Our results show that by extracting Keilin and Hartree's oxidase preparation (*Proc. Roy. Soc. London, B*, 125: 171; 1938) with 4 per cent sodium desoxycholate, it is possible to obtain a preparation which resists centrifugation for 3 hours at 20,000  $\times$  g and which is clear to the naked eye. Such a preparation is 2.5 times as active as the original Keilin and Hartree suspension. A partial purification has been effected by fractionating with 2 per cent and then 4 per cent of sodium desoxycholate to yield a preparation with 5 times the activity of the original suspension.

Several lines of evidence indicate the presence of cytochrome oxidase: (1) reduced cytochrome c is readily oxidized by small amounts of the enzyme preparation; (2) cytochrome c is a necessary constituent for the oxidation of hydroquinone; (3)  $1 \times 10^{-3}$  M sodium cyanide and sodium azide inhibit the oxidation of hydroquinone; (4) a mixture of 95 per cent carbon monoxide and 5 per cent oxygen inhibits the hydroquinone reaction by 66 per cent in the dark, and light causes an almost complete reversal of the inhibition.

*Synthesis of lipids from proteins in Colpidium campylum.* CHARLES G. WILBER AND GERALD P. SEAMAN.

The problem of the synthesis of protein, carbohydrate and fat is a fundamental one in general physiology. Much work has been done on the problem in the vertebrates; very little

on the protozoa. From the work of Mast and Pace we know that the flagellate *Chilomonas paramecium* has amazing powers of synthesis. It is also well known that lipids occur in ciliates in the form of visible globules. The types and origin of these lipids is still uncertain. Conflicting results have been obtained using the staining reactions of Nile blue sulfate. An investigation was, therefore, undertaken to ascertain the kinds and the formation of lipids in the ciliate, *Colpidium campylum*. The organisms were grown in sterile, fat-free proteose-peptone solution. Five days after inoculation, samples were taken and tested for cholesterol (*Jour. Biol. Chem.*, 24: 227; 1916), fatty acid (*Jour. Biol. Chem.*, 77: 53; 1928), and phospholipid (*Jour. Lab. Clin. Med.*, 16: 158; 1930). No cholesterol was found. In a culture containing 44,000 colpidia per cc. there was 3.4 mg.% phospholipid; 2.5 mg.% was present in the cell-free culture medium. In a culture of like population there was 232 mg.% fatty acid; 183 mg.% was present in the cell-free culture medium. The results indicate that the colpidia do not synthesize cholesterol but that they produce very large amounts of fatty acid. This latter result is in accord with work done on bacteria and on rhizopods. It is suggested that fatty acid is formed in *Colpidium campylum* from the deaminated amino acid via pyruvic acid and acetaldehyde (by repeated condensations) and that glycerol is formed via pyruvic and lactic acids, glycogen, and glyceric aldehyde.

## JULY 29

J. T. BONNER. No abstract submitted.

B. LIBET. No abstract submitted.

OTTO MEYERHOF AND JEAN R. WILSON. No abstract submitted.

## AUGUST 5

*Irreversible differentiation in certain plant cell lineages.* R. BLOCH.

In ground parenchyma of the stem of *Ricinus communis* secretory cells occur the contents of which give the reactions of tannins and unsaturated fat. They appear early within the meristem of the apical growing point and, once differentiated, continue to multiply as the stem elongates. Unlike most plant tissue cells, they retain their specific physiological character during growth and division and transmit it to the daughter cells. This behavior is also evident when old internodes are wounded: the secretory cells resume growth and division, but do not dedifferentiate as other plant cells do under such conditions. They produce their own type only and exhibit stability of cell character, resembling in this, many animal cells which show similar persistence along somatic cell lineages, irrespective of external conditions.

One possible explanation of such stability of the differentiated state has been the assumption of determinants in the nature of self-duplicating cytoplasmic proteins (plasmagenes). These or their precursors could in the present case appear and come into control in certain cells as a result of differential, polar cell divisions during which cytoplasm or cytoplasmic constituents become unequally distributed or segregated.

The occurrence of such cells and of such a suggested mechanism may play a significant role in the elaboration of cellular differences in plant tissue patterns. Many cellular differences have previously been shown to arise as a result of polarity, a basic protoplasmic factor. Many others are directly induced by external physical and chemical factors in the environment which act differentially on cells in different positions in the plant body. To these may be added possible qualitative, stable changes in the cytoplasmic reactivity systems of some of the cells, accounting for some types of specific behavior.

*Alkaline phosphatase activity in early development of amphibians.* E. J. KRUGELIS.

The physical and physiological correlation of alkaline phosphatase activity and nucleic acids indicated from several different lines of research suggested investigation of this correlation in the developing amphibian embryo whose content and distribution of ribose nucleic acid

has been thoroughly studied and described by Brachet (*Embryologie Chimique*, Liège, 1945). Using the Gomori technique, the enzymatic hydrolysis of phosphate from the substrates, sodium glycerophosphate, ribose nucleic acid, and adenylic acid was investigated in developmental stages of *Rana*, *Axolotl*, and *Ambystoma*. In the growing oöcyte, the enzyme activity is concentrated in the germinal vesicle, while the basophilia indicating ribose nucleic acid is in the cytoplasm. During the late cleavage stages, the enzyme reaction becomes intense in the cytoplasm of the cells of the animal pole, exhibiting the same gradient as is found for ribose nucleic acid. During gastrulation there appears a cytoplasmic and nuclear gradient from ectoderm to mesoderm to endoderm, which is similar to the one for ribose nucleic acid. Thus it appears that alkaline phosphatase is independent of ribose nucleic acid in oöcyte stages, but becomes associated with it during late cleavage. This is supplementary evidence for the hypothesis of Brachet that during development the cytoplasmic basophilic granules of ribose nucleic acid become increasingly complex by becoming associated with various enzymes, one of which is alkaline phosphatase. This increasing complexity of association of various chemical substances is probably related to morphogenesis.

J. R. SHAVER. No abstract submitted.

AUGUST 12

D. E. COPELAND. No abstract submitted.

*Factors influencing the expression of "position-effects."* TAYLOR HINTON.

*Drosophila melanogaster* was used in a study of the factors causing variation in the expression of a second chromosome inversion, Inversion (2LR)40d. The presence of this inversion results in different degrees of disarrangement of the facets of the eyes, mottling of the pigment, and deposits of a black tumor-like substance on the surface of the eyes. It was found that the sex of the individual had no significant effect upon the phenotypic expression of In(2LR)40d. The effect of different temperatures was studied and it was shown that in flies raised at 15° or 28° the eyes were almost normal; while the eyes were most affected at 23°. Crosses were made which demonstrated that the age of the parents had no influence upon the expression of the inversion. A significant difference in degree of effect was found when offspring raised in a non-crowded, well yeasted bottle were compared with offspring raised in a crowded shell vial. The former had almost normal eyes while the latter showed a marked eye effect. Females carrying a Y-chromosome in addition to the normal complement of chromosomes showed little eye effect with the inversion while their normal brothers were significantly more affected. When the inbred stock of In(2LR)40d was crossed to certain laboratory stocks the offspring showed no change in the expression of the inversion, but when crossed to other laboratory stocks a striking increase in the expression was noted. This suggests that some stocks carry genetic modifiers which enhance the expression of In(2LR)40d while others do not. Experiments are in progress to attempt to localize and study the nature of these modifiers. It can be concluded that the "position-effect" can be altered in expression by temperature, starvation, extra Y-chromosomes, and by genetic modifiers.

D. WRINCH. No abstract submitted.

AUGUST 19

*Induced morphological changes in Tetrahymena vorax.* C. LLOYD CLAFF.

*Tetrahymena vorax*, a Protozoan ciliate previously known as *Glaucoma vorax*, is normally found in Nature as a small tailed form, varying in size from 50 to 75 microns, with an oral opening just large enough to engulf its food, bacteria.

Kidder, Lilly, and Claff reported (*Biol. Bull.*, 78, Feb. 1940) the effect of putting *Tetrahymena gelei* in association with *Tetrahymena vorax* in the same culture. Within 24 hours drastic form changes took place in the *Tetrahymena vorax*: (1) the oral opening enlarged,

(2) a large preparatory vacuole continuous with the oral opening formed, (3) the animal enlarged to a size of 100 to 250 microns, (4) it became a Carnivore and the *Tetrahymena geleii* became its prey.

It was thought it would be of interest to place a cellophane membrane between *Tetrahymena vorax* and *Tetrahymena geleii* to see if the factor responsible for this change could be shown to be diffusible.

Culture flasks were prepared as follows: Erlenmeyer flasks were modified with an extra port on the side. A Pyrex tube, enlarged at the bottom was inserted with a cellophane membrane tied securely around the enlarged flange. These were sterilized with 10 cc. of Pyrex Distilled Water in the flask, and 2 cc. of water in the tube. Then they were inoculated on one side of the membrane with Sterile *Tetrahymena vorax* grown in 2 per cent Proteose Peptone broth; and Sterile *Tetrahymena geleii* on the opposite side.

Control flasks were inoculated with *Tetrahymena vorax* on one side of the membrane and Sterile Distilled Water on the other side.

Sterility tests were routinely carried out.

The flasks were assembled on a shaker device as shown—and shaken intermittently over a period of from 24 to 72 hours.

Carnivores, or in this case Cannibals were produced in large numbers in the cultures where *Tetrahymena geleii* were introduced on the opposite side of the membrane.

No Carnivores, or in some cases only one or two Carnivores were present in the cultures where Sterile Distilled Water was present on one side of the membrane. These Carnivores were small, and did not seem to have the ability to feed properly, in as much as they contained only one or two prey, whereas the induced forms would have as many as fifty prey.

To date only 58 experiments have been performed, so this is in the nature of a preliminary report. Some interesting facts for further investigation have been disclosed.

1. The addition of particulate Yeast Harris to the cultures of vorax seems to inhibit the formation of Carnivores.

2. The addition of the bacteria *Aerobacter* seems to favor the formation of Carnivores.

3. There is evidence that there is some factor from the *Tetrahymena geleii* culture which is diffusible through the cellophane membrane which stimulates the formation of Carnivores.

PAUL S. GALTISOFF. No abstract submitted.

### *Bermuda sea urchins and their eggs.* ETHEL BROWNE HARVEY.

The common species of sea urchin near the Bermuda Biological Station are: (1) *Lytechinus variegatus* (*Toxopneustes*), usually brown in Bermuda, always white in Beaufort. Decorates itself with shells, sea weed, etc. It is ripe in spring and early summer. (2) *Triplaneustes esculentus* (*Hipponoë*), a very large (5-6 inches diameter) species with short white spines. It is ripe in early summer and early winter. (3) *Echinometra lacunata*, an oval reddish or brownish urchin living in holes in the reefs and known as the "rock" or "reef" urchin. It is ripe in late summer and early fall. (4) *Centrechinus antillarum* (*Diadema setosum*), having very long (sometimes 10 inches) sharp spines. This is ripe in summer and early winter. (5) *Eucidaris tribuloides*, a grayish urchin with blunt club-shaped spines, known as the "slate-pencil" urchin. Another species found near the laboratory but rare is *Echinoneus cyclostomus*, a very small urchin (one inch long), somewhat oval, cream colored with bright red tube feet. There is also a sand dollar, *Mellita sericeoperforata* (now *Leodia*) found at some distance from the laboratory.

The living eggs of *Lytechinus* are beautifully clear and many of the phenomena of fertilization and cleavage can be distinctly seen. The asters show well, but the spindle fibers cannot be observed until late anaphase and telophase, when the "interzonal" fibers can be clearly seen. The eggs of *Triplaneustes*, *Echinometra* and *Diadema* are very granular, and the granules obscure the nuclear phenomena.

In *Lytechinus*, the stratification with centrifugal force is: yolk, clear layer, mitochondria; sometimes clear layer also at the bottom; there is usually no oil cap; in *Triplaneustes*: sometimes oil, yolk, mitochondria, clear layer; sometimes oil, clear layer, yolk, mitochondria; in *Echinometra*: oil, clear layer, mitochondria, yolk. *Lytechinus* and *Triplaneustes* break apart into a large nucleate fraction and a small heavy fraction.

The pluteus of *Lytechinus* has a single rod in the postoral arms, with a spiny base. The pluteus of *Tripneustes* has a lattice-like skeleton in the postoral arms, united at the base and not spiny. The pluteus of the hybrid *Lytechinus* ♀ × *Tripneustes* ♂ is maternal in the form of skeleton.

### *Spermatogenesis in sphecoid wasps.* P. W. WHITING.

In the formation of male gametes plants exhibit no meiotic phenomena, since their haploid generation has no heritage of meiosis. The half-dozen invertebrate groups characterized by haploid males, however, exhibit, to greater or less extent, remnants of spermatocyte conditions presumably characterizing their diploid ancestors. In the hymenopteran male, meiotic phenomena are elaborate and are characteristically similar in many of the superfamilies from the lower plant-feeders to the aculeates. This "orthodox" plan consists in an abortive first division, not dividing the nucleus, and an equal second division resulting in two functional sperm cells. The bees, Apoidea, deviate in that the second division is unequal cytoplasmically so that only one spermatid functions. It has now been shown that in four species of sphecoid wasps there is but one meiotic division. This produces two equal spermatids. At the end of the growth period before this division takes place, there develops close to the nucleus, a body resembling a second nucleus of small size. This body becomes very conspicuous and persists through the meiotic division, occupying a position *within* the cytoplasm at one side of the cell. These phenomena are tentatively interpreted as vestiges of the first division still further reduced than in the bees and the vespid wasps. In their equal second division these species resemble the latter group following the "orthodox" plan.

## REPORT ON THE LALOR FELLOWSHIP RESEARCH

J. BRACHET

Arbacia eggs at various stages of development have been collected in large amounts for a cytochemical and chemical study of both types of nucleic acids: some have been fixed in Zenker's and Serra's fluids for a study of nucleic acids and amino-acids distribution, using if possible quantitative microphotometric methods. Most of the material collected will be used for chemical analysis of the phosphorus, ribose, ribodose and purines content of the fertilized eggs, the blastulae and the plutei; this analytical work is already under its way.

Eggs and embryos treated with lithium chloride and sodium thiocyanate, in order to produce morphological defects, have also been preserved with the hope of establishing correlations between these abnormalities and the nucleic acids metabolism.

Arbacia and Psammechinus eggs have been centrifuged at various speeds and preserved in fixatives for further study of the nucleic acids and proteins.

Other experiments have shown that in sea-urchins' eggs, as in adult tissues, a large proportion of the ribonucleic acid is linked to small, ultracentrifugeable particles.

Some time has also been devoted to the study of the slides made by Dr. E. Krugelis in order to demonstrate the localization of alkaline phosphatase in developing marine Invertebrates' eggs; her results have been presented at the M.B.L.'s scientific meeting.

Finally, an abundant material has been collected with the purpose of studying the localization of ribonucleic acid and proteins in regulative and mosaic eggs: these included Asterias, Chaetopterus (normal development and differentiation without cleavage), Mactra (normal and centrifuged), Cynthia (normal and centrifuged) and Fundulus eggs and embryos.

It is proposed to study in the nearest possible future the material which it has been possible to collect during the summer months.

### *Comparison of the binding ability of hemocyanin and serum albumin for organic ions.* I. M. KLOTZ, A. H. SCHLESINGER AND F. TIETZE.

The presence of hemocyanin in a dispersed form in the plasma of invertebrates suggests that this protein may act not only as a respiratory pigment but also as a transport vehicle for small ions. A series of studies has been made, therefore, on the binding of several organic dye ions by the serum of *Limulus polyphemus*.

In buffered solutions near pH 5 hemocyanin has been found to form complexes with the organic anion, methyl orange, though with an affinity about one-tenth that observed with the same dye and serum albumin. Thus at a concentration of free anion of about  $1 \times 10^{-4}$  molar,

approximately  $0.5 \times 10^{-5}$  mole and  $6 \times 10^{-5}$  mole, respectively, of methyl orange is bound by one gram of hemocyanin and one gram of albumin, respectively.

No significant binding of the cationic dye, chrysoidine, by *Limulus* serum was observed even at concentrations of cation of  $4 \times 10^{-4}$  molar. A series of experiments with serum albumin under comparable conditions indicated appreciable binding though less than that observed with methyl orange and albumin. If the same decrease in binding in going from the anion, methyl orange, to the cation, chrysoidine, holds in hemocyanin as is found with albumin, it would require more precise techniques than were available to detect the chrysoidine complex of hemocyanin. The quantitative data on the binding of chrysoidine by albumin indicate that 16 is the maximum number of binding sites available on the protein molecule and that the first chrysoidine complex has a binding energy of 5,000 calories/mole as compared to 5,960 calories/mole for the corresponding complex with methyl orange.

These experiments indicate that hemocyanin acts not only as a respiratory pigment in the blood of invertebrates but that it also serves in the distribution and conservation of organic ions among various organs and tissues, though its effectiveness in these respects is less than that of serum albumin.

*The production of diabetes in the toadfish with alloxan.* ARNOLD LAZAROW AND JACK BERMAN.

Alloxan was injected into 50 toadfish (*Opsanus tau*) as a 10 per cent aqueous solution. The sugar content of the blood was determined by the Folin Malmros micro method. Serial sugar determinations were carried out on blood obtained by cutting the tail fin of the fish. The normal blood sugar value of the toadfish, which depends upon the nutritional state, varied from 26 to 86 mgs. per 100 cc. (The mean of 37 determinations on 20 fish was 54 mgs per 100 cc.)

When alloxan was injected into toadfish the effect depended upon the dose and mode of administration. Of twenty animals injected intraperitoneally with doses of alloxan ranging from 300 to 1,000 mgs. per kilogram of body weight, only two showed blood sugar values greater than 100 mgs.% 48 hours after the injection. Histological examination of the tissues showed extensive necrosis of the liver. This apparent selectivity of alloxan for the liver (following intraperitoneal injection) may be related to the hepatic portal circulation inasmuch as a large part of the alloxan injected goes directly to the liver.

By contrast, subcutaneous administration of alloxan produced a blood sugar response in a much greater per cent of the animals. Of six toadfish injected with a dose of 400 mgs./kg., three showed a 24-48 hour blood sugar value greater than 100 mgs.% Of nine animals given 600 mgs./kg., six showed a 24-48 hour blood sugar value greater than 100 mgs.%; whereas eight of ten fish injected with a dose of 800 mgs./kg. showed elevated blood sugars at 24-48 hours. The 24-48 hour blood sugar value of animals considered diabetic ranged from 100 to 328 mg.%. Although a number of the injected animals died after 24 to 72 hours, some were kept alive for 5 days and showed a persistence of their hyperglycemia.

The urine obtained from the bladder of several fish was also examined for sugar. In two fish whose blood sugars were 200 and 268 mgs./100 cc. the urine sugar concentration was 73 and 146 mgs.% respectively.

Since the islet tissue of toadfish is segregated into discrete organs apart from the digestive pancreatic tissue, direct chemical studies can be carried out on this material. Such a study is not possible in mammals where the islet tissue constitutes a very small fraction of the pancreas. Investigations on the sulfhydryl content of the islet tissue of toadfish before and after alloxan administration will be carried out.

*Localization of adenosinetriphosphatase (ATP-ase) in the giant nerve fiber of the squid.* B. LIBET.<sup>1</sup>

It is conceivable that a basic feature of nerve impulse conduction, the permeability change in the membrane, depends upon a structural change in certain membrane proteins. If the latter resemble the myosin system of muscle, they should be associated with ATP-ase activity.

<sup>1</sup> This work was carried out under a Fellowship of the Lalor Foundation, and was also supported in part by a U. S. Navy contract to the University of Chicago for research in nerve metabolism.



To test this hypothesis, measurements were made of the ATP-ase activity of the axoplasm, extruded from cleaned giant axons of *Loligo pealii*, and of the remaining sheath portion. With the tissue homogenized in 0.58 M KCl, and the activity measured in 0.58 M KCl, 0.003 M  $\text{CaCl}_2$ , veronal buffer (pH = 7.4) and 0.003 M ATP, axoplasm splits phosphate from ATP at the rate of about 0.2  $\gamma\text{P/mg. wet weight}$  (in 30 minutes at about 26.5° C.), while the average figure for the sheath is 19, about 100 times as much.

Not only is the ATP-ase activity of the nerve fiber almost exclusively confined to the sheath portion, but the latter has an activity even greater than that of squid muscle. Contrary to the case of cholinesterase, which is also localized in the sheath (D. Nachmansohn and B. Meyerhof, *Jour. Neurophysiol.*, 5: 348-361; 1941), ATP-ase is not more concentrated in the optic ganglion than in the nerve.

The nerve ATP-ase also resembles vertebrate muscle ATP-ase in its substrate specificity (it did not appreciably attack hexosediphosphate, muscle adenylic acid, or glycerophosphate, and these hydrolyses were not stimulated by  $\text{Ca}^{++}$  addition). Its response to addition of varying  $\text{Ca}^{++}$  concentrations was also similar.  $\text{Ca}^{++}$  removal from the tissue by citrate or oxalate strongly inhibits the residual activity. However, nerve ATP-ase responded considerably less than muscle ATP-ase to the sulfhydryl poisons, 0.001 M p-chloro-mercuric-benzoate, chloropicrin, or p-carboxy-phenyl-arsenoxide.

Work has been initiated in the direction of attempting to make more purified preparations of ATP-ase from nerve tissue, so that the physical and chemical properties of this enzyme can be more fully compared to the myosin-ATP-ase system.

#### *An analysis of d-amino acid oxidase in invertebrate tissues.*<sup>1</sup> CLAUDE A. VILLEE.<sup>2</sup>

In the course of work on the development of a chemically defined medium for *Drosophila* it was found (Schultz, *Anat. Rec.*, 96: 540; 1946) that d-amino acids seemed to be toxic to the larvae whereas l-amino acids permitted normal growth. Since the two types of isomers are metabolized by different enzyme systems in mammals, one possible explanation of this fact is that *Drosophila* lack d-amino acid oxidase. Experiments using dl-alanine as a substrate were unable to demonstrate the presence of the enzyme in ground *Drosophila* larvae (Villee, unpublished data). Studies were then made to see if this was a general phenomenon among invertebrates. The enzyme was measured manometrically using the Warburg constant volume respirometer, dl-alanine as the substrate and with the tissue suspended in a 0.05 M phosphate buffer solution at pH 7 or pH 8 (Rodney and Garner, *Jour. Biol. Chem.*, 125: 209; 1938). The tissues used were suspensions of sea urchin sperm, sea urchin eggs, teased muscle fibers from scallop adductor muscle, spider crab claw and tail muscle, lobster claw muscle, whelk foot (white) and radula (red) muscle, and homogenates of starfish digestive gland and whelk liver. None of these tissues, from animals of three different phyla, showed any d-amino acid oxidase activity and it is possible that the lack of this enzyme is a general characteristic of invertebrates.

#### *A study of nucleoproteins from invertebrate tissues.* CLAUDE A. VILLEE.<sup>3</sup>

Nucleoproteins have been extracted previously from vertebrate tissues, primarily calf thymus and fish sperm, and from plant tissue, wheat germ, yeast and bacteria. The present study was undertaken to see whether nucleoproteins could be extracted from invertebrate tissues and how their properties compared with those from other sources.

Nucleoproteins were extracted by two different methods, strong salt (2 M NaCl) solution or 0.5 per cent desoxycholate solution, from squid testis, spermatophore sac, sperm receptacle and ovary; sea cucumber testis and ovary; sea urchin sperm and eggs; starfish testis, ovary and liver; whelk testis and chaetopterus sperm. All of these tissues gave viscous solutions on extraction and these, on purification and precipitation in distilled water or alcohol, gave white fibers. The most viscous solutions were obtained from extracts of squid testis and spermatophore sac; these exhibited strong birefringence of flow. When examined in the Beckman spectrophotometer, these solutions showed typical nucleoprotein absorption spectra, with a maxi-

<sup>1</sup> I am indebted to Mr. C. Lloyd Claff for the Warburg apparatus used in these experiments.

<sup>2</sup> Lalor Research Fellow.

<sup>3</sup> Lalor Research Fellow.

mum at 2,600 Å. The amount of nucleoprotein not extracted by strong saline and extractable by desoxycholate is negligible. Nucleoproteins from marine animals differ from vertebrate nucleoproteins in being extracted by 2 M NaCl but not by 1 M NaCl. Analyses were made for total phosphorus, desoxyribonucleic acid phosphorus, ribonucleic acid phosphorus and phosphoprotein phosphorus by the method of Schmidt and Thannhauser and for ribonucleic acid and desoxyribonucleic acid by the method of Schneider.

The nucleic acid-protein complex of all the nucleoproteins used is dissociated in both strong salt solution and in desoxycholate solution. *Loligo testis* nucleoprotein in 2 M NaCl was dialyzed against 2 M NaCl for a week. At the end of that time the outer saline gave positive results with the ninhydrin and chloroform gel tests for protein, so the squid testis nucleoprotein contains a low molecular weight, dialyzable protein, presumably protamine. The nucleic acid-protein complex may be separated by the chloroform-octyl alcohol procedure of Sevag. In one experiment this procedure was repeated four times with 24-hour shaking periods each time. The resulting nucleic acid was practically free of protein; the last chloroform extract contained only minute amounts of protein. The separated nucleic acid fibers and protein powder have been washed thoroughly with alcohol and ether and dried in vacuo and will be used in further chemical studies on the nucleic acid and protein moieties by means of filter paper chromatography.

# PAPERS PRESENTED AT THE MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

SEPTEMBER 8 AND SEPTEMBER 9

FIRST SESSION—H. F. BLUM, CHAIRMAN

*Production of a wave of negativity by acetylcholine at a phase boundary (model of the nerve impulse).* T. C. BARNES.

The negative phase boundary potential generated by acetylcholine at the interface between oil (representing the lipid layer on nerve) and saline (representing tissue fluid) affords a possible explanation of the negative potential associated with activity in the nervous system (see Barnes and Beutner, *Science*, 104: 569; 1946). It was formerly believed that phase boundary potentials were direct current potentials giving a permanent shift in base line. Recent experiments indicate that a concentration of electrogenic substance too low to establish a permanent potential will set up a transient wave at the oil interface. Thus 0.05 mg. acetylcholine in 0.07 cc. saline is placed at the interface between guaiacol and saline generates a 3 mv. wave of negativity lasting 0.2 second. The equilibrium concentration in the 200 cc. of saline is 1 in 6 million. These waves can be repeated many times by adding more increments of acetylcholine. If the acetylcholine is added in a set-up with no oil layer there is no detectable electrical effect. These signals have been recorded on a Garceau, Rahm and Grass electroencephalograph, Cambridge electrocardiograph and by a cathode ray oscilloscope and D.C. amplifier (through the kindness of Dr. H. K. Hartline).

Controls of added saline have little or no effect, KCl gives a much smaller negativity than acetylcholine but tetraethylammonium iodide is more than twice as active as acetylcholine.

The one microgram of acetylcholine per gram present in living nerve could produce a wave of negativity if released directly on the lipid surface.

*The interrelation of the chloride excreting cell (gill) and the pseudobranch of Fundulus heteroclitus.* D. EUGENE COPELAND.

Evidence of a chloride excreting cell type in the gill filaments of *Fundulus heteroclitus* is presented. The cell is columnar and usually reaches from the basement membrane to the free surface of the gill. In sea water conditions there is typically present an oval vesicle at the distal end of the cell. It opens by a small pore to the exterior. Chloride tests ( $\text{AgNO}_3 + \text{HNO}_3$ ) give a very strong response in the vesicle and less so in the body of the cell. In fresh water conditions the vesicle is always absent. Chloride tests give a response in the cytoplasm of the tip of the cell, often giving a bar-like effect. In both conditions the cell is rich in fine mitochondria.

Observations of the gland-like pseudobranch are presented. In sea water condition the cells are typically filled with heavy, rod-like mitochondria oriented parallel to the long axis of the cell and to the free (vascular) surface of the cell. The rods are grouped parallel to each other, giving the cell an appearance somewhat like striated muscle. Preliminary results show a progressive granulation of the mitochondria in the fresh water adapting animal, reaching a peak at about twenty-four to forty-eight hours, then returning to a rod-like condition almost like that found in the sea water condition. In sea water adapting animals there is progressive rod-like organization, reaching the typical sea water picture in about twenty-four hours. Three lines of evidence suggest a functional relationship of the pseudobranchial and chloride cells. (1) Homology. Both cells are acidophilic. Leiner (1938) has demonstrated vesicles in the pseudobranchial cells of *Smaris alcedo*. (2) Leiner (1938) has localized carbonic anhydrase in pseudobranch. This might aid in the Cl shift in the chloride cell. (3) Differential behavior of mitochondria described above may show a participation in adaptation.

*Some physiological factors involved in the maximum velocity at which trout swim.*  
KENNETH C. FISHER.

It is well known that trout tend to orientate against a current of water and that they will swim actively so as to maintain themselves in a constant position. If the current is gradually increased, a point is reached above which the fish cannot swim fast enough to maintain the constant position. The physiological factors which determine this limiting velocity are under investigation.

Rogers showed, some years ago in this department, that the velocity varies with temperature and, in fact, that it is a maximum at about 10–15° C., far below lethal temperatures. We find now that the frequency of "spontaneous" movements made by fish in still water also varies with temperature and shows a maximum at 10–15° C. Furthermore, the limiting velocity for fish in a current may be varied in a simplified environment by changing the light intensity. It is, therefore, tentatively concluded, that the limiting velocity is determined, in the first instance, by the frequency of swimming movements; that the latter occur due to "spontaneous" or other stimuli and may then vary in frequency or in strength in accord with the strength and frequency of these stimuli; and finally, that the response to the stimuli received is modified in accord with the ambient temperature by the activity of the body surface temperature receptors.

*Protoplasmic injury substances and their effect on the organism.* L. V. HEILBRUNN AND LOUIS MOREAU (by invitation).

Earlier work from this laboratory has shown that when tissues of various organisms are injured, substances are produced which have a thrombin-like action in causing the clotting of protoplasm in the absence of calcium. We believe that the production of thrombin-like or thromboplastic substances by injured tissues may help to explain some of the baffling problems of clinical physiology. In earlier experiments we used the protoplasm of sea-urchin eggs to test for the presence of thrombin-like substances. In recent work we have used blood clotting times as an indicator. For this purpose the frog is a useful experimental animal. When frog tissues are injured, the frogs show typical shock reactions, but the problem of studying these shock phenomena is comparatively uncomplicated, for stoppage of respiratory movements or even stoppage of circulation has little immediate effect. We have developed methods for studying the clotting time of frog blood. When frogs are subjected to violent head injury, the blood, when examined immediately, shows shortened clotting time, indicating the release to the blood of thromboplastic substance. Some minutes later, there is a counter reaction and the clotting time of the blood is prolonged. Injury due to electric currents can also produce changes in the blood clotting time. Injury substances may be released from the brain or from the muscles. If the muscles of the legs are injured by electric currents, the effect on the frog as a whole may be inhibited by ligaturing the upper thighs. We have begun to work also on heat injury. In general, the results indicate strongly that various types of tissue injury release thrombin or thromboplastic substances to the blood stream, and that these substances can affect tissues throughout the organism.

*Preparation and properties of cation-permeable erythrocytes.* M. H. JACOBS AND MARIAN WILLIS.

It has long been recognized that unrestricted permeability of an erythrocyte to both anions and cations would be osmotically incompatible with its continued existence. The view has more recently been expressed by several workers, particularly by Wilbrandt, that certain so-called "non-osmotic" hemolytic agents may act through the creation of a condition of cation permeability, resulting in an osmotic unbalance between the erythrocyte and its surroundings. The present paper is concerned with the mode of action of one such hemolytic agent, *n*-butyl alcohol, which shows in a particularly clear and uncomplicated manner some of the consequences of an induced cation permeability.

Erythrocytes having different chosen degrees of porosity, as measured by a series of non-electrolytes of increasing molecular volume, may be prepared by exposure for different lengths

of time to a solution of 6 volumes per cent of butyl alcohol in isotonic NaCl, sucrose, or both, followed by dilution with and, if desired, by subsequent washing in a pure sucrose or sucrose-NaCl solution. The cells so prepared obey very satisfactorily predictions made from newly derived equations for the theoretical ionic and osmotic equilibria between cation-impermeable and cation-permeable cells and solutions containing NaCl alone, sucrose alone, and both substances together.

Among the theoretical properties of cation-permeable cells shown by erythrocytes treated with butyl alcohol are the following: (1) unlimited swelling in all concentrations of NaCl, (2) strong and rapid shrinkage in pure isosmotic sucrose solutions, (3) assumption of definite intermediate volumes in most mixtures of NaCl and sucrose, with protection against hemolysis by extremely low concentrations of sucrose, (4) more rapid swelling rather than shrinkage on alkalization of an external NaCl solution, and (5) shrinkage followed by swelling beyond the original volume on the addition of concentrated NaCl to an appropriate external mixture of NaCl and sucrose.

*Evidence of active transfer across the human erythrocyte membrane.* PAUL G. LEFEVRE.

Following an earlier report concerning the effectiveness of various sulfhydryl inhibitors in delaying hemolysis of human red cells in isotonic glycerol, the course of the volume changes upon addition of glycerol to suspensions of these cells in isotonic salt or sugar solutions was followed in the Parpart apparatus. The inhibitory action of cupric and mercuric ions, iodine, and p-chloromercuribenzoate upon the rate of entry of glycerol, and the reversibility of this inhibition by sulfhydryl groups, was thus verified. Similar inhibitory effects upon the exit of glycerol from cells previously equilibrated with glycerol solutions was observed. The glucoside phlorizin, usually considered inhibitory to phosphorylations, exhibited a similar effect; chemical methods, however, indicated that no inorganic phosphate was consumed in the accumulation of glycerol into these cells.

The slow swelling of erythrocytes in solutions containing glucose was also delayed by phlorizin, and was particularly sensitive to mercuric ion and to p-chloromercuribenzoate, although cupric ion showed no inhibitory effect whatever. Comparison of the volume changes upon addition of various increments of glucose to suspensions of cells in isotonic saline showed that the rate of swelling was inversely proportional to the sugar concentration at the higher concentrations, but followed the usual exponential form with the lower concentrations; this set of relations indicated that there was a limiting rate at which glucose could enter these cells, regardless of the magnitude of the concentration gradient. Previous equilibration of the cells with glucose at 0.05–0.1 M greatly delayed the subsequent entrance of glucose when the external concentration was raised; this suggests that the postulated transfer mechanism functions only when the intracellular concentration is below a certain critical concentration in this range. These results have been partly duplicated with galactose or levulose in place of glucose, and have been verified by chemical analyses of the glucose content of the suspending medium.

*Phosphatase activity in the chloride cells in the gill of Fundulus heteroclitus.* OLIVE PETTENGILL.

A histochemical examination of the chloride cells of the gills of *Fundulus heteroclitus* which had been adapted to both fresh water and salt water was made, using the Gomori technique. Material was fixed in cold 80 per cent alcohol or acetone, dehydrated and embedded in the usual manner. The substances used which gave a positive reaction were yeast adenylic acid, yeast nucleic acid, hexose diphosphate and the standard, beta-glycerophosphate.

There is a strong activity in the fresh water gill, localized generally at the distal end, between the nucleus and the end of the cell, and the cell seems to be enlarged. In sea water, the vesicle, which is characteristic of this condition, is surrounded by a very dark ring of activity, the signet ring effect. The total amount of activity is less than that seen in fresh water.

Copeland (1947) has presented evidence to support the theory that this cell does excrete chloride in the salt water fish. One might conclude that the dark ring of phosphatase activity

around the chloride-excreting vesicle may indicate an increase in metabolic activity necessary to transport the chloride from the cell into the vesicle. This would constitute a physiological mechanism to remove chloride from the body.

Krogh (1939) suggests that euryhaline fish such as *Fundulus* may have a double mechanism in the gills that would pump chloride out of the body under sea water conditions and into the body under fresh water conditions. It is possible that the two mechanisms are represented in a single cell. The strong phosphatase activity in the fresh water form may indicate a reversal of the functional polarity of the cell with consequent pumping of chloride into the body. The increased amount of phosphatase activity may be indicative of the greater osmotic gradient encountered in the fresh water condition.

## SECOND SESSION—A. K. PARPART, CHAIRMAN

### *Permeability to radioactive sodium in frog eggs.* WILLIAM R. DURYEE AND PHILIP H. ABELSON.

It is the main purpose of this paper to show how much and how fast radioactive sodium ( $\text{Na}^{24}$ ) exchanges with normal sodium ( $\text{Na}^{23}$ ) in a typical vertebrate egg. Secondary purposes are to show where the sodium goes inside the cell and to throw some light on the regulative mechanism. Permeability has heretofore been considered chiefly a membrane phenomenon. Our results indicate that permeability to sodium is really a more complex matter in that diffusion through cytoplasm and blocking of internal exchange are the main limiting factors. Due to high activity of the  $\text{Na}^{24}$  plus sensitive counters results can be expressed accurately to  $\pm 1 \times 10^{-8}$  grams Na.

Ovarian eggs of *Rana pipiens* were cut apart and exposed to various Ringer solutions made up with  $\text{Na}^{24}$ . Control solutions were made with identical amounts of normal sodium. The  $\text{Na}^{24}$  was made by bombardment in the cyclotron. Our criteria of actual penetration were three-fold: (a) by radio-autographs of sections of eggs that had been frozen in liquid air, (b) by puncturing the cell to remove aliquots of the yolk-cytoplasmic mass for direct measurements with a Geiger counter, and (c) by isolation of the cell nucleus for direct measurement.

A marked difference was found between the up-take curves for live and dead cells. With the former radio-sodium rises rapidly in concentration to a value of  $2.5 \times 10^{-7}$  gm. Na in 30 minutes and tends to level off. At the end of 4 hours the average concentration is  $3.7 \times 10^{-7}$  gm. Na per egg. In the case of dead cells  $\text{Na}^{24}$  diffuses in more rapidly reaching a concentration of  $38 \times 10^{-7}$  gm. in 30 minutes and then levels off. These "terminal values" correspond roughly with the total sodium per egg found by direct chemical analysis of the ovaries. It is concluded that all the sodium in a dead egg is exchangeable, the membrane forms practically no barrier and Na once in the egg diffuses freely. In a live egg only 10 per cent of the total sodium is exchangeable.

From washing experiments with live eggs it is clear that there are two kinds of sodium in the cell—one freely mobile and the other slowly exchangeable over a period of ten or more hours. Calcium-free or double calcium Ringer solution did not affect the initial rate or quantity of exchange of  $\text{Na}^{24}$ , but after several hours a deficiency in calcium permits an exchange of sodium to a level of  $6 \times 10^{-7}$  gm. as compared with a level of  $3 \times 10^{-7}$  gm. Na per egg immersed in a Ringer with twice the amount of calcium.

From radio-autographs it is evidence that distribution of sodium is relatively uniform throughout the cytoplasm in less than 30 minutes, but that it is strikingly concentrated inside the nucleus. This shows that the nuclear membrane is readily permeable to  $\text{Na}^{24}$  and that the amount of sodium available for exchange is approximately twice as great in the nucleus as in the cytoplasm.

### *Accumulation and distribution of radioactive materials in goldfish.* C. LADD PROSSER.

In experiments at the Metallurgical Laboratory, University of Chicago, the uptake of products of uranium fission and of radioactive sodium by goldfish was measured. Accumulation of

radioactive strontium, barium, rare earths and sodium was rapid initially (during the first few hours of immersion) and continued at a rate greater than the loss of radioactivity in the medium by decay and adsorption. The ratio of the concentration of active material in the fish to that in the solution increased with time. This concentration ratio was usually between 10 and 100. The concentration ratio was independent of dose over the range investigated ( $0.01\mu\text{C}$  to  $1.0\mu\text{C}/\text{ml.}$ ).

Calcareous tissues, such as skeleton and scales, accumulated about 75 per cent of the radio-strontium and radio-barium in the whole fish. Rare earths were accumulated in the visceral tissues, especially the intestine, and were more strongly adsorbed on feces and glass than the alkaline earths. The comparable distribution, particularly of bone-seekers, on immersion and injection, indicated that these elements were taken up through gill and oral membranes and were distributed by the blood. Radio-sodium was present in greater proportion in the gills at six hours than later. Some fission elements, particularly the rare earths, appeared to be precipitated along the intestinal folds. The yolk of eggs in ripe ovaries accumulated rare earths but not strontium or barium.

Young growing or regenerating bone accumulated more radioactive material than did older bone; cartilage accumulated none of the bone-seekers. Uptake of  $\text{Sr}^{89}$  was greater when the surrounding pond water was low in calcium (1.8 p.p.m.) than when it was high in calcium (36 p.p.m.). Stable strontium also retarded the uptake of radio-strontium but to a less degree than calcium.

When the fish were transferred from active to inactive pond water they failed to lose their radioactivity faster than by decay. The rate of loss of radio-strontium appeared to be unaffected by the calcium content of the water. Goldfish were unable to remove appreciable quantities of fission elements from suspended clay.

### THIRD SESSION—J. H. BODINE, CHAIRMAN

#### *A method for subcooling cells under microscopic observation at room temperature.*

ROBERT CHAMBERS AND SILVIO BEAZ (*by invitation*).

The principle of the technique is to cool the objective of the microscope so that the objective serves as the refrigerator to sub-cool an oil-enclosed hanging drop, containing the specimen, suspended from a coverslip which roofs the micromanipulation chamber on the stage of the microscope.

The cooling is accomplished by means of a closed, circular, metal trough placed around the objective, and communicating with a tank of compressed gas. The inner wall of the trough is perforated with holes to permit the expanding gas to blow on the objective.

The chamber on the stage of the microscope is hermetically sealed off and the movements of a microneedle in the chamber are made possible by having it pass through a mercury-filled five to ten mm. hole in a brass plate which walls off the end of the chamber directed toward the micromanipulator.

An essential feature is to prevent fogging of the optical system of the microscope. This is accomplished by having drierite ( $\text{CaCl}_2$ ) in a trough inserted into the lower end of a wide-barrelled drawtube of the microscope. The front lens of the objective is kept dry by the escaping gas rushing about it. The space within the closed chamber is also dried to minimize the formation of water condensation on the hanging oil drops.

The temperature is regulated by varying the rush of the gas and by operating an electric microheater consisting of a platinum or nichrome strip of wire in the chamber joining the ends of two copper wires connected with a dry battery cell.

The temperature is determined by the congealing of one or more of several drops of oil-mixtures suspended in the chamber. The temperature at which the congealing occurs has been previously determined by preparing paraffin wax ( $50^\circ\text{C}$ . melting point) dissolved in Nujol oil at different percentages.

The hanging drops enclosed in oil cool below their freezing points and freezing is accomplished by "seeding" the drops or the enclosed cells by means of the tip of a microneedle.

Preliminary experiments on cells indicate that the icicles which form make a pattern in accordance with the structure in a living cell.

*The possible role of peroxidase in cellular respiration.* DAVID R. GODDARD AND ANNE M. SLATER.

The respiration of horse radish root sections is inhibited by low concentrations of cyanide, azide, and hydroxylamine; and photoreversibly by carbon monoxide. The inhibition results are identical with those obtained with cytochrome oxidase plants, but extracts fail to show any catalytic activity with cytochrome c and hydroquinone, and cytochrome oxidase is presumably absent. The experiments appear to establish the respiratory role of peroxidase in this tissue, in which the peroxidase is functional with molecular oxygen, that is, it functions as an oxidase. The carrier is unknown, but is not dioxymaleic acid, as this compound brings about an extra oxygen consumption of one atom per molecule of added substrate. The dioxymaleic acid oxidase is probably an artifact.

*Resistance of cell membranes to internal pressures.* PETER RIESER (introduced by D. V. Heilbrunn). READ BY TITLE.

In connection with other studies from this laboratory on the magnitude of the osmotic forces to which a cell membrane is exposed, an attempt has been made to determine in general how much pressure the membranes of cells can withstand without rupturing. Accordingly, cells were microinjected and the pressure created by the injection was determined. In order to accomplish this, a dial-type pressure gauge was connected to the injection circuit in close proximity to the micro-pipette. Eggs of the sea-urchin *Arbacia punctulata*, the starfish *Asterias vulgaris* and the annelid worm *Chaetopterus pergamentaceus*, as well as isolated muscle fibers of the frog *Rana pipiens*, were used in the experiments. The plunger of the microinjection syringe was pushed slowly either by hand or with the aid of a screw device, and the injection fluid—sea-water for the marine eggs and Ringer's fluid for the muscle fibers—was forced out of the pipette. In order to provide for ready visibility, the injection fluids were colored with methylene blue. When the pressure became too great for the cell membrane to withstand, the membrane ruptured. Typically the break was immediately sealed by the surface precipitation reaction. There was rarely any large volume increase of the cell. When the cells ruptured, the pressure was read off on the gauge. The values on the gauge ranged from 40–175 mm. Hg. Actually, these pressures recorded on the gauge are higher than the pressures acting within the cell, for the flow of fluid through the pipette would tend to reduce the pressure existing at the tip of the pipette. Thus one may conclude that for various types of cells the pressure necessary to rupture the cell membrane is typically less than 40–175 mm. Hg. It is hoped later developments will give more exact rather than maximum values.

*The effect of temperature change on the oxygen consumption of yeast.* F. G. SIHERMAN.

The effect of changing the temperature on the rate of oxygen consumption of a bottom yeast (American Type Culture Collection No. 9080) was determined with a Warburg respirometer. The yeast was grown aerobically at 30.1° C. for 24 hours in a modification of the medium described by Nickerson and Carrol (1943). After harvesting, the yeast was washed three times with M/15  $\text{KH}_2\text{PO}_4$  and resuspended in this buffer. Glucose was tipped in at zero time from the side arm of the vessels. Controls were run at 30.1° C. Experimental vessels were placed in the 30.1° bath for two hours and then removed to the cold bath for 24 hours after which they were returned to the warm bath. A thermobarometer was transferred with the vessels when they were removed from one bath to the other. Three series of experiments were run with the warm bath at 30.1° C. and the cold bath at 5.2° C., 10.6° C. and 14.8° C., respectively. The control and experimental vessels were run in quadruplicate in each experiment.

In every case the yeast which had received cold treatment began to consume oxygen at the same rate as the controls when they were returned to the warm bath. In no case was an overshoot or undershoot observed in this yeast under the conditions of these experiments.



*Studies of the metabolism of normal and mutant imaginal discs of Drosophila.*  
 CLAUDE A. VILLEE.

A basic problem of physiological genetics is how do embryonic tissues which are going to develop into different organs differ in their enzyme constitutions. The imaginal discs of *Drosophila*, discrete groups of cells developing into particular adult structures, provide a means of attacking this problem. One approach in analyzing the effects of a gene in development is to determine its effect on metabolic activity as reflected by the rate of oxygen consumption. This has been done by means of the Cartesian diver ultramicrorespirometer. Previous work (*Proc. Nat. Acad. Sci.*, 32: 241; 1946) showed that normal wing discs have a  $QO_2$  of 20; "miniature" wing discs, a  $QO_2$  of 18; and "vestigial" wing discs, a  $QO_2$  of 9. The leg discs of all three stocks have a  $QO_2$  of 20. On the hypothesis that each gene regulates a particular biosynthesis, a search for the enzyme affected by the "vestigial" gene has been made by adding specific inhibitors or substrates to the buffer solution containing the disc in the diver. Normal and "vestigial" disc respiration is inhibited by cyanide and azide and thus is mediated by an iron or copper porphyrin system, probably the cytochromes. Respiration is also inhibited by the hydroxynaphthoquinones SN 5949 and SN 5090, believed to inactivate some enzyme between cytochromes b and c. The respiration of "vestigial" discs is increased to that of normal discs by ascorbic acid and to a lesser extent by p-phenylene diamine and hydroquinone. These experiments indicate that the respiratory enzymes of the developing imaginal discs of insects are similar to those of a wide variety of cells from bacteria to mammals, that the cytochrome oxidase system of "vestigial" is normal and that the "vestigial" gene affects some enzyme below cytochrome c in the respiratory chain.

The oxygen consumption of the eye discs of wild type, Bar, and Double Bar larvae have been measured similarly; at two hours before pupation their uptakes are 5.7, 7.3, and 8.2 ml. $\mu$   $O_2$ /hr./disc, respectively. The Bar and Double Bar discs are slightly smaller than wild type, so the differences on a  $QO_2$  basis would be even more striking. Thus, in contrast to m and vg wings, where the structures with smaller adult sizes have a lower  $QO_2$  in the imaginal disc, the B and BB eye discs have a higher rate of oxygen consumption than the normal eye discs although the resulting eyes are smaller than normal. The addition of 0.02 M succinate to the substrate increases the oxygen consumption of all three types but the greatest increase occurs in wild type: 14.2, 10.2, and 8.8 ml. $\mu$   $O_2$ /hr./disc for wild, B and BB, respectively. The eye disc respiration is also inhibited by cyanide, azide and the hydroxynaphthoquinones.

*Lipid metabolism in the protozoa.* CHARLES G. WILBER AND GERALD R. SEAMAN.

It is well known that protozoa contain fat in the cytoplasm and there is some evidence that fat can be synthesized by these organisms. Identification of fat has previously depended on staining reactions with Nile blue sulfate or with Sudan dyes. In order to study the synthesis of fat in ciliates, sterile cultures of *Colpidium campylum* were grown on fat-free proteose-peptone for various periods of time. Lipids were estimated by standard biochemical methods: fatty acid by oxidation, phospholipid and cholesterol colorimetrically. The population of the cultures reached a maximum of about 65,000 colpidia/cc., 48 hours after inoculation; it remained constant until the fourth day and then rapidly decreased. At all times the amount of fatty acid and of phospholipid remained constant in the organisms themselves. Fatty acid in the cell-free culture fluid increased linearly until the 4th day, remained constant until the 6th day, and finally increased to a maximum of 313 mg./100 cc. on the 8th day. Phospholipid in the culture fluid increased linearly until the 3rd day; from the 3rd to the 4th day there was a tremendous increase in phospholipid from 1.6 mg. lipid P/100 cc. to 3.8 mg. lipid P/100 cc. The phospholipid then remained constant until about the 6th day after which it increased to 5.3 mg. lipid P/100 cc. on the 8th day. At no time was cholesterol found in the organisms or in the culture fluid. The results indicate that colpidia can synthesize lipids from purely protein sources. There is also evidence the sudden increase in phospholipid foreshadows the decrease in population.

It is suggested, as a working hypothesis, that the so-called "growth substance" reported by various workers is lipid in nature. Whether cholesterol, which has been reported to be carcinogenic in action, influences the growth in *Colpidium* is not at present known. Experiments are in progress to ascertain the effect of cholesterol and of other lipids on metabolism and growth in *Colpidium campylum*.

# THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

## THE RESPIRATORY QUOTIENT OF *DROSOPHILA* IN FLIGHT

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### INTRODUCTION

In view of the unusual performance of insect flight muscles, which are able at times to make over a million successive contractions at rates up to several hundred per second, information on the biochemical transformations which supply the energy for this intense activity is of special interest. The purpose of this study has been to ascertain what evidence as to the types of substrate utilized during flight could be obtained from continuous measurements of the respiratory quotient.

The earlier students of the respiration of flying insects, whose results have been reviewed in detail by Jongbloed and Wiersma (1934), were generally content to assume a respiratory quotient of 1. Jongbloed and Wiersma were the first to provide an experimental basis for this theory when they allowed individual bees to fly for periods of about 5 minutes in closed containers and showed that oxygen was consumed and carbon dioxide given off in nearly equal amounts. Beutler (1936 a, b; 1937) then demonstrated that bees and some other Hymenoptera were dependent upon sugar for the ability to fly, and in fact for the maintenance of life, but because of the specialized physiology of bees and their adaptation to the food stores of the hive one is hesitant to transfer these findings without further support to other types of insects.

Nevertheless there is evidence that in the Diptera also the flight respiratory quotient is 1. Thus, Chadwick and Gilmour (1940) in two short flights of 5 and 6 minutes duration found with *Drosophila repleta* an equivalence of oxygen uptake and carbon dioxide output, while Williams and colleagues (1943) were able to show for *D. funebris* and *Lucilia sericata* a quantitative correspondence over a period of 60 to 90 minutes between the rate of disappearance of glycogen and the amount of flight activity as measured by the rate of wing-beat and the duration of flight. Whether carbohydrate was the only fuel consumed during these longer flights was not determined. Studies of flight respiration by Davis and Fraenkel (1940) and by Krogh and Zeuthen (1941), the most recent known to the writer, were concerned primarily with other aspects of the problem, and do not afford a basis for answering this question. Therefore, since the technique of Fenn (1928) provides a means of making continuous simultaneous measurements of oxygen consumption and carbon

\*These experiments were done while the writer was a member of the Department of Physiology of the University of Rochester School of Medicine and Dentistry.

dioxide production, it seemed worthwhile to combine this method with stroboscopic determinations of the rate of wing-beat in order to study possible fluctuations in the respiratory quotient as related to activity, particularly in the later stages of prolonged flights.

#### MATERIAL AND METHOD

The procedure followed differed little from that described by Chadwick and Gilmour, except that the Warburg manometers of their experiments were replaced by a differential volumeter with side tube for conductivity measurements, of the type devised by Fenn. The volume of each vessel was approximately 30 ml.; the capillary was 30 cm. long and had a capacity of about 5 cu.mm. per cm.  $\text{CO}_2$  given off by the insect was absorbed in 3 ml. of 0.01–0.02 M  $\text{Ba}(\text{OH})_2$ , which covered the bottom of the experimental vessel and was tipped into the side tube for measurement of the change in impedance at intervals of 1 minute while flight was in progress. Simultaneous records of  $\text{O}_2$ -consumption were obtained from the movement of an index drop in the capillary which connected the control and experimental vessels, while wing-beat frequency was measured stroboscopically, as described previously, at 10-second intervals.

The sensitivity of the measurements is estimated as follows:

$\text{CO}_2$	: $\pm 0.02$ cu.mm.	(= 1 scale division on slide wire of conductivity bridge)
$\text{O}_2$	: $\pm 0.025$ cu.mm.	(= 0.1 mm. on capillary scale read with aid of a magnifying glass)
wing-rate	: $\pm 1$ per cent	(manufacturer's specification for stroboscope)

Relatively greater errors were introduced by imperfect synchrony between the three types of measurement, since, for example, a lag of 30 seconds between recordings of oxygen and carbon dioxide would place the second observation in excess by 50 per cent of the respiratory rate during the last minute of the period of measurement. The observers gave special attention to eliminating this source of error in so far as possible, so that when the measured amounts of oxygen or carbon dioxide have been summed for periods of 5 or more minutes, the uncertainty from this cause may be estimated conservatively at not more than 2 per cent of the totals.

It is more difficult to take account of lag in the absorption of carbon dioxide under the conditions of the experiments, where an insect of not more than 1.5 mg. weight in a vessel of 30 ml. capacity was using oxygen and producing carbon dioxide at variable rates up to nearly 1 cu.mm per minute. Absorption curves were determined for the vessel and showed that when a few cu.mm. of  $\text{CO}_2$  were introduced 93–95 per cent was absorbed in 5 minutes, but these could give only an approximation to the actual experiments, the purpose of which was to follow continuously the unknown and unpredictably changing rate of gaseous exchange of the animal. Since the rate of absorption will be proportional at any instant to the concentration of gas existing in the vessel, identical quantities of carbon dioxide liberated over a given interval of time will give different percentages of absorption by the end of the period, if the lag in absorption is appreciable, depending on whether the rate of liberation was constant, rising or falling. Thus one would need independent knowledge of the rate of change in rate of  $\text{CO}_2$ -production in order to

apply satisfactorily any absorption "constants" which had been determined for the system. For this reason, no attempt has been made to correct the data presented below.

Lag in absorption of carbon dioxide not only introduces an asynchrony between the record of respiratory exchange and the measurements of wing-beat frequency when sudden changes occur, but may result also in spurious values for the respiratory quotient. Consider, for instance, the consumption of 100 units of oxygen and the production of 90 units of carbon dioxide during an interval in which only 70 per cent of the carbon dioxide produced is absorbed. Readings taken at the beginning and end of this period would indicate a carbon dioxide production of only 63 units; the remaining 27 units of this gas are still in the vessel, however, and occupy space vacated by a similar number of units of oxygen. Consequently the volumeter records the disappearance of only 73 units of oxygen. The apparent respiratory quotient for the period will therefore be given by the ratio 63:73 ( $= 0.86$ ), whereas the true value is 0.90. Thus, the apparent respiratory quotient is depressed below the true value, if this is less than 1, and elevated above it, if it is really greater than 1. In many experiments lag in absorption will not lead to much error in the estimation of the respiratory quotient, since, if the rate of production of  $\text{CO}_2$  remains constant, the concentration of the gas in the vessel and consequently the rate of absorption will rise, so that in course of time the apparent respiratory quotient will approach the true value. But in experiments with insect flight, it is often impossible to allow time for equilibration after changes in the rate of production of  $\text{CO}_2$ .

Evidently, if there had been no other factors to be considered, it would have been preferable in this study to have used vessels small enough to make the lag in absorption of  $\text{CO}_2$  insignificant. But to have done so would have required rather drastic changes, which have not yet proved feasible, in the technique of the conductivity measurements. The data obtained with the larger vessel are therefore presented here and are to be interpreted with appropriate reservations, especially for periods in which marked changes occurred in the respiratory rate, such as at the beginning and end of flight.

As specimens for the study, adult males of known age were taken from cultures of *D. virilis* and *D. americana*, which were kindly supplied by Dr. H. D. Stalker. The animals were reared in the laboratory (temperature  $20^{\circ}$ – $25^{\circ}$  C.) in half-pint bottles on a standard medium. In preparation, the flies were immobilized with ether and the dorsal tip of the abdomen fastened with paraffin to a paper mount which was later affixed to the stopper of the respirometer vessel. Only one specimen was run at a time. The animal rested in the head-down position in the vessel, with the feet in contact with a light spring platform which could be retracted by means of an electromagnet situated outside the water bath in which the respirometer was immersed. Control measurements were made in order to ensure that the respiration recorded was that of the animals rather than the apparatus.

All experiments were run at a bath temperature of  $20.0 \pm 0.01^{\circ}$  C. After a half-hour for equilibration, the resting respiratory exchange was followed for a considerable length of time (see Table I) before the animal was stimulated to fly by withdrawing the platform on which his feet were supported. Flight continued until it ceased spontaneously, after which the resting exchange was again measured

for a number of hours during the post-flight period. The animal was weighed at the conclusion of the experiment.

These experiments would not have been possible without the interest and co-operation of Professor Wallace O. Fenn, in whose laboratory they were carried out. I am also indebted to my wife for assistance with the measurements.

TABLE I  
*Preflight respiratory exchange in Drosophila*

Specimen number	Weight	Age	Duration of measurements	Average CO <sub>2</sub> -output	Average O <sub>2</sub> -uptake	Average R.Q.
	mg.	days	min.	cu.mm./gm./min.		
1	1.5	0-2	120	39.7	26.8	1.48
2	0.9	2	188	31.5	25.1	1.25
3	0.9	2	71.5	48.5	32.2	1.51
4	1.0	2	148	40.0	29.2	1.37
5	1.0	5	1460	20.6	27.6	0.75
6	0.7	2	253	35.6	32.1	1.11
7	1.1	2	102	33.4	28.7	1.16
8	0.9	2	181	30.8	19.9	1.55
9	1.5	2	380	23.2	18.8	1.23
10	1.2	3	369	28.6	23.1	1.24
11	1.4	2	294	28.1	25.0	1.13
12	1.1	5	367	25.3	18.2	1.39
13	1.5	7	369	20.8	22.1	0.94
14	1.2	2	191	37.2	35.2	1.06
Average				31.7	26.0	1.23

Specimens numbered 12, 13 and 14 were males of *D. virilis*; the others, males of *D. americana*.

## RESULTS

### *a. Respiration before flight*

Data on the respiratory exchange during the period before flight are given in Table I, in which the age and weight of the specimens are also recorded.

The average rate of oxygen consumption (26.0 cu.mm. per gm. per min.) is in good agreement with resting values which had been determined for *Drosophila* by other methods (Kucera, 1934; Chadwick and Gilmour, 1940). Comparable figures for production of CO<sub>2</sub> seem not to have been published previously.

### *b. Respiration during flight*

Of the 14 flies listed in Table I, 6 flew continuously for periods of 56 to 154 minutes. The respiratory data obtained have been summed for each of these individuals over successive intervals of approximately 20 minutes, and are shown together with the corresponding average rates of wing-beat and the calculated respiratory quotients in Table II. The correlation between rates of wing beat and of respiration is illustrated graphically for 4 of these animals in Figure 1, where each point plotted represents approximately 5 minutes of flight. The average rate of oxygen consumption during these long flights was about 14 times, and of CO<sub>2</sub> production about 11 times the previous resting rate; in the earlier study with *D. repleta* the average oxygen consumption during flight was 13 times the value at rest.

TABLE II

*Respiratory exchange of Drosophila in successive periods of longer flights*

Specimen number	Duration of measurements min.	Average frequency beats/min.	Total CO <sub>2</sub> -output cu. mm.	Total O <sub>2</sub> -uptake cu. mm.	Average R.Q.
1	22	9208	17.14	16.09	1.06
	22	9125	16.39	17.68	0.93
	21	8669	13.75	14.34	0.96
	22	6927	8.82	9.31	0.95
3	21.5	9233	8.21	7.17	1.14
	20	7892	5.81	5.82	1.00
	20	7580	5.82	5.61	1.04
	9.3	7004	1.95	2.32	0.84
10	20.3	9345	14.48	13.89	1.04
	20	8721	11.67	11.36	1.03
	22	7860	10.68	9.98	1.07
	21.3	7827	10.13	10.45	0.97
	21	7729	9.29	9.77	0.95
	20.5	8065	10.88	11.03	0.99
	20.2	8181	11.10	11.69	0.95
	8.8	8201	4.97	4.66	1.07
11	19.0	9111	11.60	11.92	0.97
	22.3	8219	12.60	12.41	1.01
	18.7	7071	7.10	7.79	0.90
	22.2	6756	7.95	8.09	0.98
	20.2	6519	7.41	7.56	0.98
	22.7	6638	7.14	8.06	0.89
	21.5	6354	6.19	6.30	0.98
13	20.2	7659	8.83	8.96	0.98
	17.5	7450	8.09	7.30	1.11
	18.0	6917	6.48	6.09	1.06
14	21.0	8289	9.90	10.29	0.96
	17.3	7128	8.59	8.69	0.99
	13.2	7869	6.52	6.78	0.96
	12.8	7632	5.87	5.94	0.99

Performance in shorter flights is depicted adequately by the data for the initial periods of those flies which flew for longer times, and was covered also in the earlier publication.

### *c. Respiration after flight*

Irregularly spaced measurements of oxygen consumption and carbon dioxide production after flight were made on the 6 individuals which flew for long periods. These have been recorded in Table III. Differences between the rates determined after flight and the average rates before flight are shown in columns 5 and 6 of the table.

TABLE III  
*Respiratory exchange of Drosophila after long flights*

Specimen number	Interval of measurement  min.	Rate after flight		Excess over rate before flight		Postflight R.Q.
		CO <sub>2</sub> cu.mm./gm./min.	O <sub>2</sub> cu.mm./gm./min.	CO <sub>2</sub> cu.mm./gm./min.	O <sub>2</sub> cu.mm./gm./min.	
1	0-5	75	128	35	101	0.58
	5-10	39	36	-1	9	1.07
	10-30	25	41	-15	14	0.60
	30-50	23	31	-17	4	0.76
	50-92	22	45	-18	18	0.47
	92-106.5	17	31	-23	4	0.56
	106.5-113.3	17	38	-23	11	0.46
	113.3-119.9	15	21	-25	-6	0.72*
	119.9-123.1	16	16	-24	-11	1.00*
3	0-4.7	161	117	112	85	1.39
	4.7-25.7	41	77	-8	45	0.54
	25.7-52.7	17	31	-32	-1	0.57
10	0-5	145	140	116	117	1.04
	5-14	53	89	24	66	0.60
	14-20	42	58	13	35	0.72
	20-31	34	36	5	13	0.96
	31-78.7	19	28	-10	5	0.66
11	0-13	66	83	38	60	0.79
	13-23	33	29	5	4	1.12
	23-43	25	24	-3	-1	1.04
	43-59	26	25	-2	0	1.04
	59-70	23	30	-5	5	0.76
13	0-8.5	110	85	89	63	1.28
	8.5-20.5	53	43	32	21	1.22
	20.5-37.5	32	29	11	7	1.11
14	0-8.3	92	87	55	52	1.06
	8.3-22.7	44	42	7	7	1.06
	22.7-50.2	32	39	-5	4	0.83
	50.2-75.2	34	38	-3	3	0.90

\* Specimen moribund.

Rates are given to the nearest whole number; R.Q. computed from actual observations.

## DISCUSSION

### *a. Respiration before flight*

The most striking feature of the preflight respiration was the high respiratory quotient, which averaged less than 1 with only 2 of the 14 individuals examined (Table I). So far as could be determined, these observations were not the result of any artefact in the experiments and at first it was thought possible that the flies might be giving off ammonia as an end product of protein breakdown. If this should occur, the volumetric determinations of oxygen consumed would yield er-

roniously low values, while there would be no interference with the absorption of  $\text{CO}_2$ . The possibility was ruled out in several ways. In some experiments (numbers 12, 13 and 14 of Table I) an acid-soaked piece of filter paper was suspended in the experimental vessel, but high respiratory quotients were found throughout most of the period nevertheless. In other experiments, individual flies were confined over acid, which was then tested with Nessler's reagent for the presence of ammonia. A few early trials gave some positive results, but later when more care was taken to exclude contamination from outside sources, even large numbers of flies failed to produce significant amounts of ammonia. The high respiratory quotients were characteristic only of the first three hours or so of measurement, and fell to values of 1 or somewhat less when the observations were carried over longer periods.

While no definite explanation can be given for these facts, which deserve further study, it is suggested that the flies, recently removed from food in the culture bottles, may have been laying down energy reserves by conversion of carbohydrate to fat. Jongbloed and Wiersma (1934) occasionally noted high respiratory quotients before flight in their experiments with bees, but were inclined to ascribe these aberrant values to experimental error.

#### *b. Respiration and rate of wing-beat during flight*

Two objectives were in mind during this phase of the experiments: (1) measurement of the respiratory exchange, and (2) a retesting of the correlation found in a previous study between rate of wing-beat and the level of the respiratory metabolism.

As may be seen from the data in Table II, very seldom did the observations indicate a respiratory quotient significantly different from 1, even in the later stages of continuous flights which lasted from 1 to 2 or more hours. Thus the conclusion reached by Williams and colleagues on the basis of glycogen determinations, that carbohydrate constitutes the chief source of the energy required for flight, receives strong support from the present observations. These workers found that 4 to 5 day old *D. funebris* when freshly removed from culture contained glycogen to the extent of 4.88 per cent of the live weight. After 90 minutes of flight only 1.30 per cent remained, so that its rate of disappearance, in terms of the weight of the animal, amounted to about 2.4 per cent per hour. Very similar results were obtained from analyses of thoracic glycogen in the blowfly, *Lucilia sericata*.

For comparison, the weights of glycogen equivalent to the  $\text{CO}_2$  which was produced in the flights of the present study have been calculated. It was assumed that complete oxidation of the carbohydrate to  $\text{CO}_2$  and water occurred, on which basis the rate of utilization of glycogen during flight amounted to from 2.0 to 3.2 per cent of the final live weight per hour (Table IV). These figures are sufficiently close to those obtained by Williams *et al.* to make it seem likely that glycogen is not merely the principal, but probably the sole source of energy consumed during flight; however, the scatter in the results is such as not to exclude the possibility that smaller amounts of other types of substrate might also have been utilized, although there is no definite evidence pointing in this direction.

An intimate correspondence between the level of activity and the respiratory rate is brought out in the plots of Figure 1, where the logarithms of the rates of  $\text{CO}_2$ -production are compared with the logarithms of the rates of wing-beat. Each



point represents the average rates over a period of approximately 5 minutes, and the rate of production of  $\text{CO}_2$  has been corrected by subtraction of the average rate found for the specimen in the preflight resting period.

TABLE IV  
*Glycogen equivalents of respiration of *Drosophila* during long flights*

Specimen number	Weight mg.	Duration of flight min.	Total wing-beats (approximate)	Total $\text{CO}_2$ produced		Calculated consumption of glycogen	
				cu. mm.	micrograms	micrograms	per cent of body weight per hour
1	1.5	87	738,000	56.10	110.0	67.5	3.2
3	0.9	71	573,000	21.79	42.7	26.2	2.4
10	1.2	154	1,268,000	83.20	163.0	100.0	3.2
11	1.4	147	1,064,000	60.00	117.6	72.2	2.1
13	1.5	56	409,000	23.40	46.0	28.2	2.0
14	1.2	64	484,000	31.29	61.5	37.7	2.9

Inspection of the graphs shows clearly that there must be some underlying linear relationship between the logarithms of frequency and respiratory rate. Similar results had been obtained during shorter flights in the majority of the experiments of Chadwick and Gilmour, who reasoned from a comparison of wing movement with simple harmonic motion that the work done per stroke should be proportional to the (frequency)<sup>2</sup>, so that a direct proportionality between (frequency)<sup>2</sup> and the oxygen consumption per stroke would be expected. Essentially the same relationship was developed from a somewhat different approach by Reed *et al.* (1942) in a study in which the characteristic rate of wing-beat under standard conditions was compared with various bodily dimensions for a number of species and races of *Drosophila*. The formula they derived,

$$(\text{wing-beat frequency})^2 = k \times \text{muscle volume} / (\text{wing area}) (\text{wing length})^3,$$

expresses the fact that the (wing-beat frequency)<sup>2</sup> increases in direct proportion to the power of the motor mechanism and in inverse proportion to the resistance to be overcome by the wings.

If the work done per stroke is proportional to the second power of the frequency, then the work done in unit time must be proportional to (frequency)<sup>3</sup>, and this quantity would be related directly to the rate of respiration, the energy input, through an efficiency factor. In the absence of satisfactory determinations of the work output of *Drosophila* in flight, the actual efficiency remains unknown, but the coefficient may be supposed to include, in addition to the overall efficiency of the biochemical transformations which supply the energy of muscular contraction, a second component expressing the efficiency with which the thorax and wings are able to translate muscular work into movement of air. A perfectly linear relationship between (frequency)<sup>3</sup> and rate of respiration would indicate that the efficiency remained absolutely constant throughout the period of flight, or that changes in efficiency were exactly balanced by shifts in amplitude or in the angle of attack.

As an aid in visualizing the actual performance, straight lines have been drawn through each of the plots in Figure 1 to conform to the equation

$$\log (\text{CO}_2/t - r) + \log k = 3 \log F, \quad (1)$$

where  $\text{CO}_2$  = total  $\text{CO}_2$  recorded during time  $t$ ,

$r$  = mean rate of production of  $\text{CO}_2$  during the preflight resting period,  
and

$F$  = average frequency during time  $t$  in wing-beats per minute.

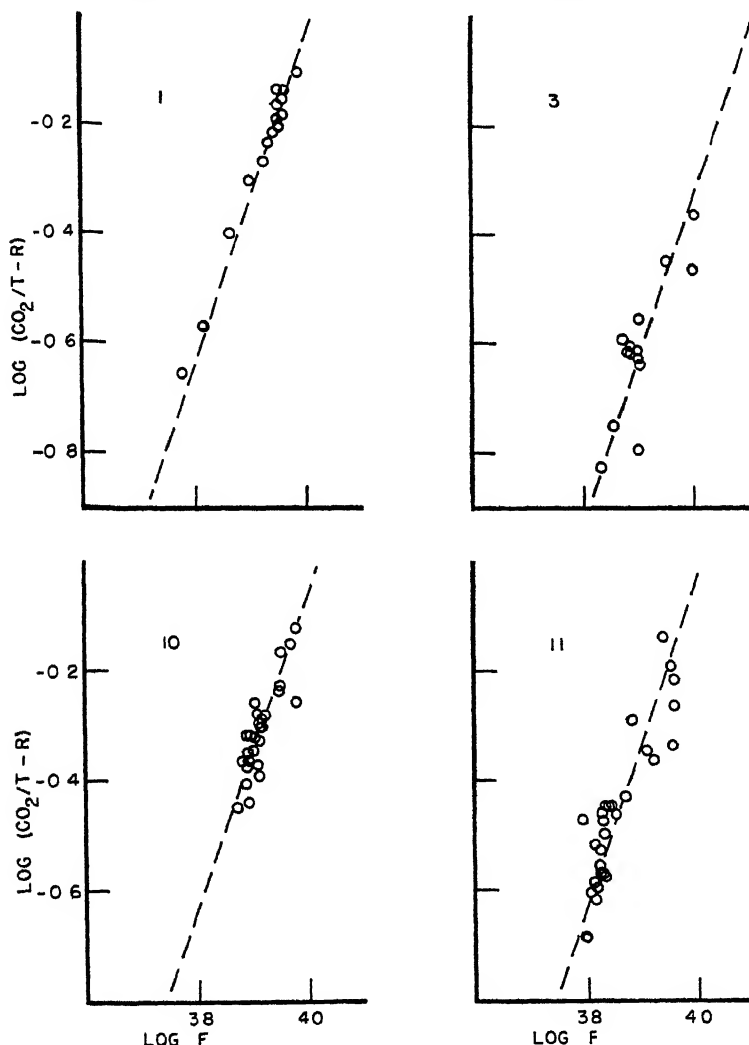


FIGURE 1.  $\text{CO}_2$ -output as a function of wing frequency in *Drosophila*. Each point represents the average rates during a 5-minute period of flight. The broken lines were calculated to conform to the equation  $\log (\text{CO}_2/t - r) + \log k = 3 \log F$ , with values chosen for  $\log k$  as follows: Specimen number 1, 12.03; number 3, 12.32; number 10, 12.05; number 11, 12.03.

The quantity  $k$  includes a proportionality factor, plus the efficiency, and probably also a component related to the bodily proportions of the individual insect, in accordance with the findings of Reed *et al.* The values of  $k$  used for determining the lines of Figure 1 were calculated for each specimen by averaging the values obtained when the paired observations of  $(\text{CO}_2/t - r)$  and  $F$  were substituted in Equation 1.

Although it is evident from the graphs that there is a fairly close correspondence between performance as predicted by Equation 1 and the actual observations, it is clear also that measurements covering a given 5-minute period may depart rather widely from expectation. These random variations are to be explained as due in part to experimental error, the result chiefly of lag effects in absorption of  $\text{CO}_2$  when rapid shifts in the rate of respiration occurred, and may be ascribed also in some measure to alterations in the amplitude of the wing stroke and in the angle of attack. Since the work output per minute would vary with the third power of the amplitude and with the sine of the angle of attack, small variations in these quantities would exert an appreciable effect. Such changes are not infrequently noticed under stroboscopic illumination, but unfortunately no satisfactory technique has been developed for their measurement. The absence, in spite of these irregularities, of any consistent trend away from the linear relationship between  $F^3$  and  $\text{CO}_2$ -output as lower rates are encountered in the later stages of flight is noteworthy, since it shows that in general the efficiency of flight is the same at this time as when the animals were fresh. This could hardly be the case if any great shift had taken place in the biochemical reactions which deliver the energy for flight; thus these observations give added support to the inference already drawn from the respiratory data: that metabolism of carbohydrate furnishes the energy utilized throughout the entire period.

### *c. Respiration after flight*

For the first 5 or 10 minutes following flight the measurements consistently yielded resting rates of respiration much higher than those found before flight (see Table IV). This is almost certainly an artefact resulting from lag in absorption of  $\text{CO}_2$  which had been given off during the flight period, and should not be regarded as evidence for an oxygen debt incurred during activity. With smaller vessels in which the lag in absorption was negligible, it was shown previously that the oxygen debt represented an amount sufficient to sustain flight for only a fraction of a minute, that it was independent of the length of flight, and that it was paid off in 2 minutes after flight had ceased.

During subsequent periods rather variable results were obtained, both as regards the respiratory level and the respiratory quotient. Experimental error was magnified at the lower rate of gaseous exchange, and in addition the activity of the specimens varied from time to time. Nevertheless, the values found for the respiratory quotient were often significantly less than 1, although the rate of oxygen consumption fluctuated for some hours within a range close to the preflight average. These observations accord with other evidence that the carbohydrate reserves are seriously depleted by extended flights.

It is obvious, of course, that flight may be, and normally is terminated by causes other than exhaustion of carbohydrate, and since some individuals were found to be able to fly for over 2 hours, there is a fair presumption that specimens whose performance fell short of this figure may have ceased flying with a considerable store of carbohydrate still in reserve. Some of the higher respiratory quotients recorded

after flight may be accounted for on this basis. The fact that other individuals survived for a number of hours with respiratory quotients of 0.70 or less points to a fundamental difference between the resting metabolism of *Drosophila* and bees, which according to Beutler succumb within a few minutes if they are allowed to fly until their carbohydrate is exhausted. The survival of these flies presents an opportunity for investigating the process of recovery under controlled feeding which could be made to yield valuable information as to the type of substances which can be converted into sources of energy for flight, a phase of the subject concerning which nothing is known at this time.

#### SUMMARY

1. Continuous volumetric measurements of oxygen consumption and conductimetric measurements of carbon dioxide production were made at 20° C. on individual specimens of *Drosophila americana* and *D. virilis*: (a) before flight; (b) during flight to exhaustion; and (c) after flight. During flight the rate of wing-beat was determined stroboscopically.

2. Before flight, the average results were: CO<sub>2</sub>, 31.7 cu.mm. per gm. per min.; O<sub>2</sub>, 26.0 cu.mm. per gm. per min.; R. Q., 1.23.

3. During flights lasting from 56 to 154 minutes the rate of respiration was approximately proportional to (wing-beat frequency)<sup>3</sup>. The rate of oxygen consumption averaged 14 times and the rate of CO<sub>2</sub>-production 11 times the previous resting rate. The R. Q. was essentially 1.

4. Variable rates of respiration were observed after flight; the R. Q. was frequently much less than 1.

5. It is concluded that carbohydrate furnishes the principal and possibly the only source of energy for flight, and that *Drosophila* are able to survive for some hours after their carbohydrate has been exhausted.

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# THE EFFECT OF ILLUMINATION AND STAGE OF TIDE ON THE ATTACHMENT OF BARNACLE CYPRIDS\*

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The attachment of barnacle cyprids to exposed surfaces is influenced by the degree of illumination and the surface color. Cyprids of *Balanus amphitrite* and *Balanus improvisus* were found by Visscher (1927) to attach in greater numbers to dark or shadowed surfaces. McDougall (1943) noted this same behavior for the cyprids of *B. eburneus*. When exposed only at night, however, Pomerat and Reiner (1942) noted that *B. eburneus* attached to black, clear and opal glass in equal numbers. Twice as many barnacles attached to the black glass in natural day-night exposures. Gregg (1945) investigated the effect of contrasting dark surfaces against lighter general surroundings. He concluded that while no correlation could be established between the degree of contrast and the frequency of attachment, "shading" was suggested as acting as a stimulus to the attachment of the larvae of *B. eburneus* to opal glass collectors.

In the present investigation the rate of attachment of cyprids of *B. improvisus* during daylight and night exposures, and the effect of artificially illuminating the collecting surface at night were observed. The effect of the tide on the rate of attachment of cyprids was also studied. This work was carried out at the boat shed of the Beach Boat Slips Corporation in Biscayne Bay, Miami Beach, Florida.

## METHODS

Four stations for sampling the barnacle cyprid population were established under and adjacent to a covered boat slip which extended 250 feet from the shore, the stations being at the outer end of the shed.

Stations A, B and C were control stations for comparison with Station D, which was illuminated at night. Stations A, D and B were spaced 55 feet apart, in that order in a north-south line paralleling the general flood and ebb movement of the tidal current. Station C was located 60 feet to the west of Station D. The conditions of natural and artificial illumination at each of these stations were as follows: In the daylight hours illumination at each of the four stations was nearly the same through the day. The intensity at each location depended on the proximity of the shadow of the shed. At Station A, daylight illumination was skylight; at Station B, the collector swung in and out of the sun and shadow of the shed with the phase

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of the tide; and Station C was in full sunlight, whereas Station D was under the shed roof and in the full shadow. At night, Station D was artificially illuminated, while Stations A, B and C were in darkness.

Collecting units consisting of four pieces of black glass (500 sq. cm. collecting area) were suspended from a float. The surface panel was horizontal while the others, at depths of 2, 6 and 10 feet, were hung in a vertical position. The 10-foot level was just above the bottom at low tide and three feet higher at high tide.

The periods of collection, day and night, were eleven hours each, from 7:30 A.M.-6:30 P.M., and 7:30 P.M.-6:30 A.M. The eleven-hour exposure permitted no significant overlapping period of dark to light or light to dark when the collectors were in the sea. The one-hour interval between exposures permitted counting, cleaning and reimmersing of the collection units.

TABLE I

*Numbers of cyprids attaching during consecutive night and day periods of eleven hours each at three stations. The figures are the sum of the collections on four panels of 500 sq. cm. area each, hung at different depths.*

Cyprid attachment night			Cyprid attachment day			Average	
Stations			Stations			Night	Day
A	B	C	A	B	C		
*394	205	306	403	614	784	302	600
418	279	272	386	897	997	323	760
463	534	778	1361	1392	2018	592	1590
434	768	497	701	666	1147	566	838
296	160	483				313	
**444	533	432	2352	1149	3564	470	2355
391	446	541	636	569	1829	459	1011
255	201	479	432	881	1092	312	802
219	168	240	676	564	1581	209	940
114	99	101				105	

\* February 11, 1946-February 16, 1946.

\*\* February 26, 1946-March 3, 1946.

Although the source of illumination at Station D remained fixed while the collector unit which hung below it rose and fell with the tide, an average distance of 5 feet from the lights to the water surface was used in calculating the value of incident illumination at the water surface and at the 10-foot depth. The incident illumination five feet from the lights was measured by a direct reading photometer and that at the 10-foot level calculated by using an absorption factor for the local waters determined with an underwater photometer.

The study of cyprid attachment relative to stage and direction of the tide was made by counting the cyprids attaching during intervals of two hours throughout a tidal cycle in daylight hours only. The collecting units were the same as those employed in the illumination experiments.

## DIURNAL VARIATION IN CYPRID ATTACHMENT

Observations on five successive nights and four intervening days in two separate series indicated that many more cyprids settled during the period of daylight, as shown in Table I. In the first set of observations, the ratio of the average of all the day collections to the average of the corresponding night collections indicated an increase of 2.1 times the number of cyprids attaching in daylight, while in the second series the ratio of day to night attachments was 3.5.

It is of interest to note that in the first set of collections when the cyprid density, as shown by total attachment day and night, was lower than the second set of collections there are examples of day collections being nearly of the same magnitude or even slightly less than the night attachments. This condition was found only at Stations A and B which were wholly or partially shaded from direct sunlight during the daylight hours. A partial explanation of the relatively high night attachments of the first set of collections may be due to the fact that these were made under the full of the moon whereas the second set were made in the dark of the moon. However, when the cyprid density was high, as during the second set of observations, no discrepancies from the diurnal fluctuation in attachment were noted. The maximum differences between night and day attachments were always found at Station C which had the maximum difference in illumination.

## VERTICAL DISTRIBUTION OF CYPRID ATTACHMENT

The vertical distribution of barnacle cyprids at Stations A, B and C showed no consistent pattern which would suggest dependence on any particular controlling factor. In the first set of collections, shown in Figure 1, the maximum numbers at Station A, both day and night, were found at the 2-foot level. At Station B, all the day collections showed cyprid peaks at the 6-foot level, whereas, at night, attachment was nearly uniform between the 2-foot level and the bottom, except for one observation. At Station C, the day collections resulted in maxima at the 2-foot level on three occasions and a 6-foot maximum on one occasion.

The second series of vertical samplings, as shown in Figure 2, were made at a period of higher cyprid density which resulted in collections of considerable magnitude for several of the eleven-hour periods. However, no consistent pattern of vertical distribution was apparent.

This erratic distribution of cyprids, both vertically and in comparison of stations, is of particular interest, in view of the relatively close proximity of the collecting units. The apparent random attachment of cyprids to the collecting units is probably due to a nonuniform distribution of cyprids in the water. Since the number of cyprids attaching does not decrease with depth, it is apparent that intensity of illumination does not control vertical distribution.

## EFFECT OF ARTIFICIAL ILLUMINATION ON CYPRID ATTACHMENT

The normal diurnal cycle, with lower attachment numbers at night, was abolished by the use of artificial illumination at night at Station D. The cyprid attachments at night under artificial illumination at Station D were greater than the daylight counts at this location and were nearly as large as the daytime collections at Station C where the intensity of daylight illumination was greatest. As shown in

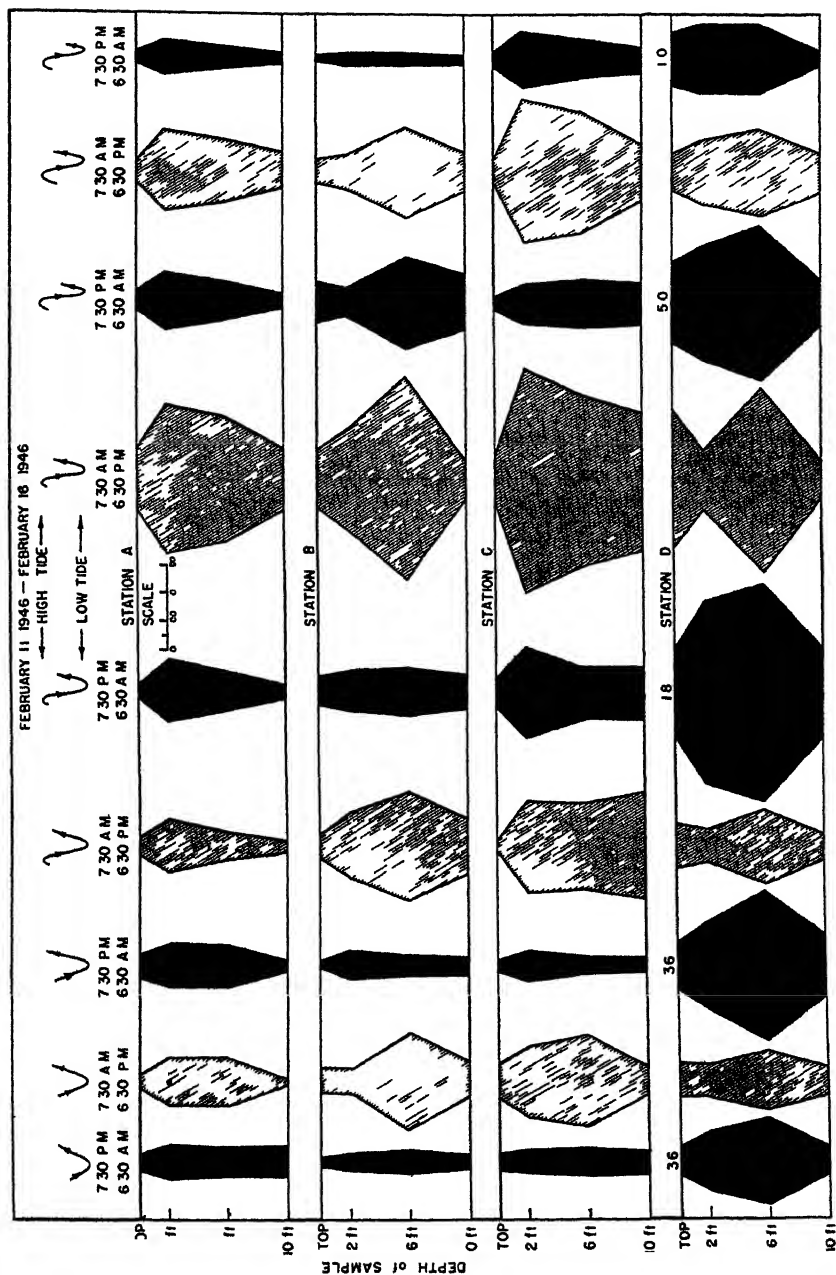


FIGURE 1. Vertical distribution of cyprids at four stations as collected in eleven hour periods, February 11, 1946–February 16, 1946. The tidal curves at the top of the figure represent the stages of the tide in which the collection took place. The numbers over the right collections of Station D are the illumination values in footcandles at the water surface used for that exposure.



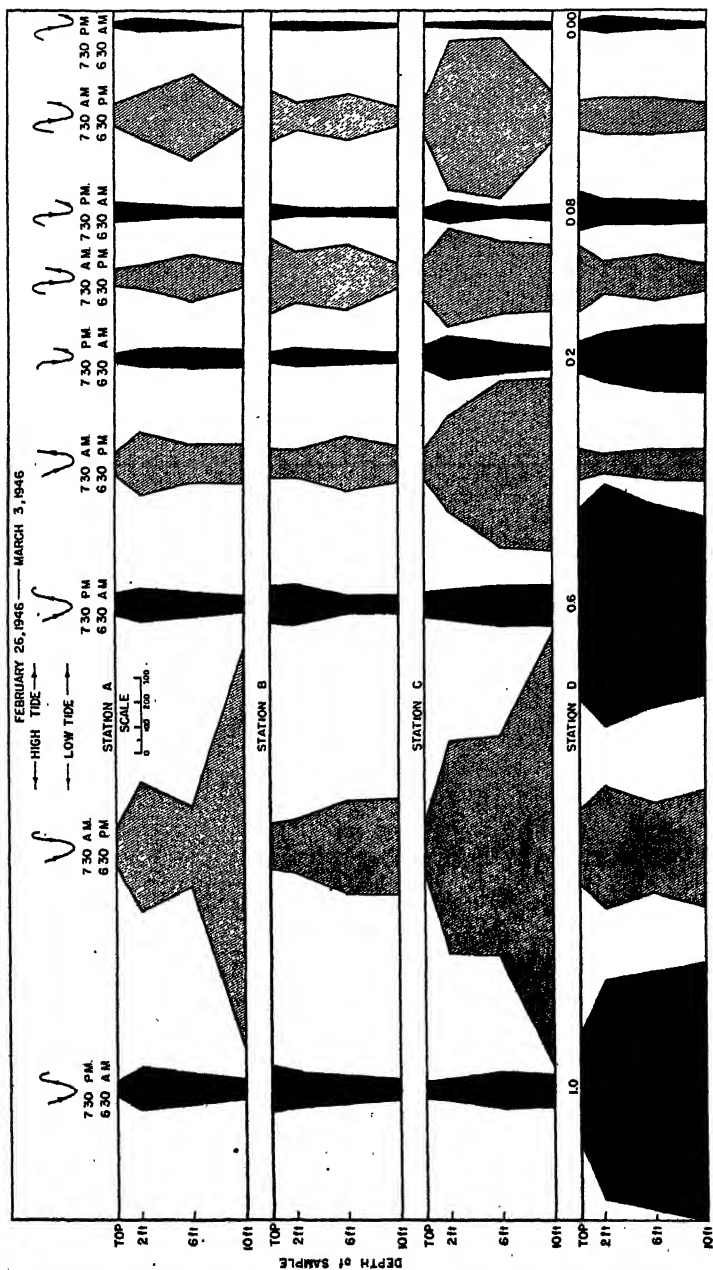


FIGURE 2. Vertical distribution of cyprids at four stations as collected in eleven-hour periods, February 26, 1946–March 3, 1946. The tidal curves at the top of the figure represent the stages of the tide in which the collection took place. The numbers over the night collections of Station D are the illumination values in footcandles at the water surface used for that exposure.

Figures 1 and 2, artificial illumination, however, had little if any effect on vertical distribution of the cyprids.

In order to determine the intensity of illumination necessary to increase the cyprid attachments at night, the illumination over the collectors at Station D was

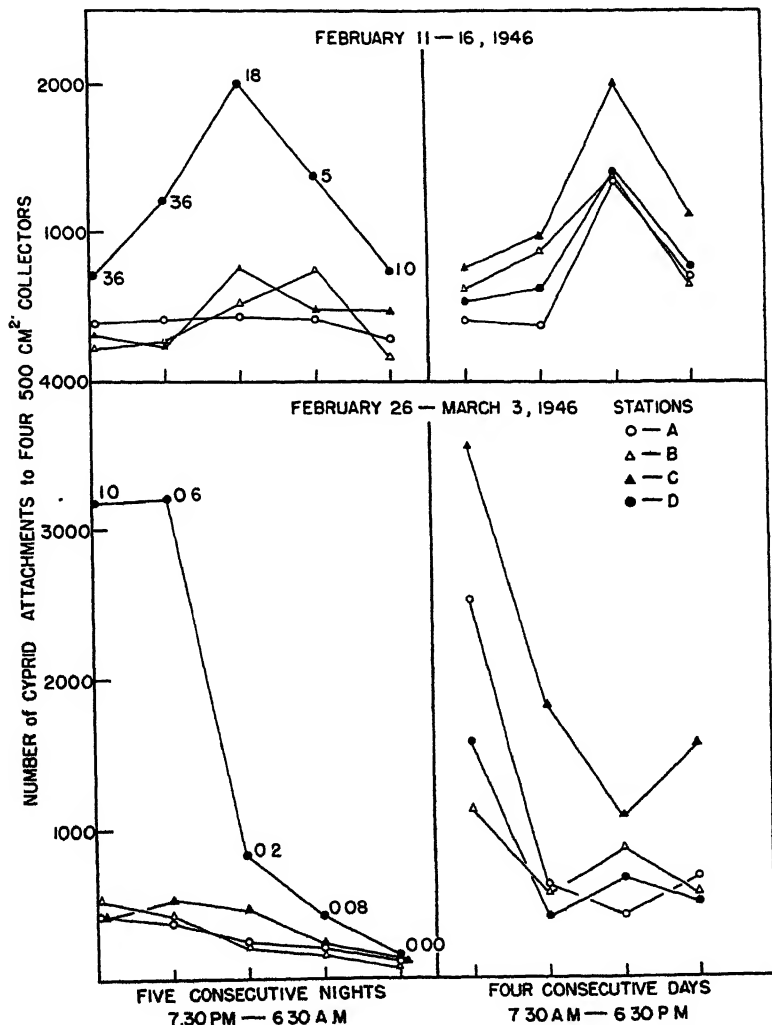


FIGURE 3. Total cyprid attachment for the eleven-hour collection periods at each station. The numbers plotted with Station D, night collections, are the values in footcandles at the water surface of the artificial illumination employed.

systematically changed. In the first series, the incident illumination at the water surface was varied from 36-1.0 footcandles, and in the second experiment, from 1.0-0.00 footcandles. The variations in vertical distribution at each station, which are apparently due to other factors, have been smoothed by totaling the cyprid accu-

mulations from each of the four glass collectors. This figure then represents the catch from the cyprid population of a vertical column of water. As shown in Figure 3, the illumination of the collectors at Station D resulted in a two to sevenfold increase in numbers of cyprids attaching over those found on the control surfaces. The daily variation in cyprid numbers attaching at night to the illuminated collectors of Station D resembled more nearly the daily fluctuation in cyprid density as found on the daylight collections of Station C. Although the night collections of Station D paralleled the cyprid density fluctuations as found at the unilluminated stations, they were of course several times greater in magnitude. This result was obtained within the limits of illumination of 36 to .08 footcandles at the water surface. On the final night exposure of the second series, when Station D was not illuminated, its cyprid collection was still slightly greater than the other three stations. However, this difference is probably within the normal variation. During the day the collections at Station D were of the same magnitude as at A and B and considerably less than those at C.

TABLE II

*Numbers of cyprids attaching in eleven hours at night at the 10-foot depth at Station D as related to quantity of artificial illumination and in comparison with the simultaneous attachment at Stations A, B and C.*

Value of illumination at D at 10-foot depth (footcandles)	Stations				D 1/3(A+B+C)
	A	B	C	D	
*5.2	110	45	87	102	1.3
5.2	38	71	56	219	4.0
2.5	45	117	184	318	2.8
.71	42	206	136	169	1.3
.14	44	34	90	64	1.1
** .14	78	92	135	1005	9.9
.09	59	71	158	710	7.4
.03	65	37	81	257	4.2
.001	38	44	66	77	1.6
.000	7	15	33	21	1.1

\* Five consecutive nights—February 11, 1946–February 16, 1946.

\*\* Five consecutive nights—February 26, 1946–March 3, 1946.

The artificial illumination, even at the 10-foot depth, consistently increased the number of cyprids attaching to the collecting surface of Station D in comparison with the control stations, as shown in Table II. The ratio of the attachment at Station D to the averages of Stations A, B and C indicates the degree of change in attachment due to illumination. The greatest differences between the illuminated collectors and controls occurred on the second and third nights of the first experiment when cyprid attachment was at a maximum. In the second set of exposures, when cyprid density was high, all the collections at the 10-foot level of Station D, even though the illumination was very low, were invariably greater than all the controls.

As indicated above, the illumination of the water surrounding the collectors was varied systematically to determine the minimum value to which the cyprid would

respond. When the cyprid density was high, thus providing more organisms which would by random selection be subjected to the contrast of luminous zone and dark area, the greatest change in cyprid attachment occurred when the illumination of the water surface was changed from 0.6 to 0.2 footcandle. At 0.6 footcandle and greater intensities, the difference in cyprid settling, in comparison to the controls, was sevenfold, while at 0.2 footcandle and less, the increase over the control col-

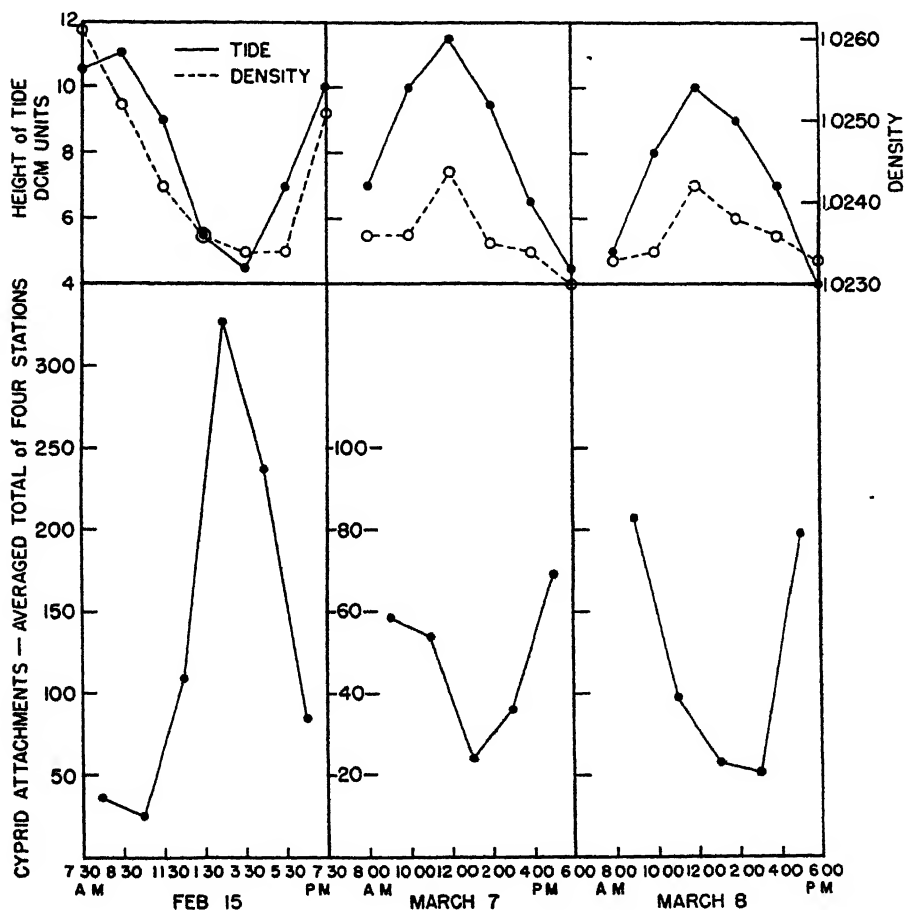


FIGURE 4. Bi-hourly cyprid attachment at each station totaled from the four collecting surfaces. The height of tide is noted in decimeter units. February 15, March 7 and 8, 1946.

lectors was only twofold. However, at illumination as low as .03 footcandle, increases in attachment over control collectors were still considerable.

Previous investigations (Visscher, 1927; Pomerat and Reiner, 1942; and McDougall, 1943) showed that the barnacle cyprid attaches in daylight in greater numbers to dark surfaces than to light surfaces. At night, as is to be expected, there is no distinction between the two colors. In this investigation illumination of dark collecting surfaces at night increased attachment. One effect of illumination

is to increase the contrast between the dark surfaces and the surrounding water. In waters with considerable suspended material, as usually found in harbors and estuaries, a beam of light is markedly scattered, producing a general illumination in the water. A piece of black glass suspended in these waters under a source of illumination was discernible to the observer as a black area surrounded by brighter water. When the water appears brighter than the collecting surface, at night because of artificial illumination, or during daylight, the cyprids may be attracted to the darker area by some negative phototropic response and may accumulate there in greater numbers than would result from chance encounters. In contrast, in the absence of light at night, the numbers of cyprids attaching may depend solely on chance encounters with the collecting surface, and are consequently lower than during periods of illumination.

#### EFFECT OF STAGE OF TIDE

On collectors exposed through the day and examined at two-hour intervals, the maximum numbers of cyprids attached during the low-tide period as shown in Figure 4. This was found whether the low tide occurred at midday, in the early morning, or late afternoon. As in the case of the longer exposures, there was no indication that the distribution of cyprids on the panels was influenced by the depth at which they were hung.

At the location of this experiment, the ebbing tide brings to the boatshed water which is isolated within the bay. It is replaced at high tide with oceanic water entering the bay on the flood. This is shown by the change in density accompanying the tidal cycle. The isolation of the bay water permits development of a large cyprid population, since few of the plankton stages are lost into the ocean prior to metamorphosis to the cyprid stage. At the low-tide period, the sampling location was occupied by the bay water with high cyprid content, and this apparently accounts for the greater attachment at that time. The high attachment at low tide cannot depend upon the lessened flow at that time, since a similar slack water occurs at high tide when the attachment is minimal.

#### SUMMARY

1. The cyprid larvae of *Balanus improvisus* were found to settle in a diurnal rhythm with maximum numbers attaching during daylight hours.
2. No consistent pattern of vertical distribution of the cyprids was found.
3. The normal diurnal cycle in rate of attachment of barnacle cyprids was nullified by the use of artificial illumination over the collecting surfaces at night.
4. The magnitude of the cyprid collection on the artificially illuminated surfaces was equal to the collection on the sun-illuminated surfaces in daylight.
5. The intensity of artificial light necessary to produce large cyprid attachments at night was of an order as low as 1 footcandle at the water surface.
6. No correlation was found between the quantity of artificial light at night and the numbers of cyprids attached.

7. The highest rate of cyprid attachment relative to the phase of the tide was found to occur when the waters of upper Biscayne Bay were sampled at the collecting station. This body of water reached the sampling station at low tide and was characterized by a high cyprid population.

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# EARLY LIFE HISTORY OF THE OYSTER CRAB, *PINNOTHERES OSTREUM* (SAY)<sup>1</sup>

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## • INTRODUCTION

The first and second zoeae of *Pinnotheres ostreum* have been described by Hyman (1924). The five crab stages parasitic in the oyster (*Ostrea virginica*) have been described by Stauber (1945). The present paper is an attempt to complete the knowledge of the development of the oyster crab from the egg to the first crab instar. This work was done at the Virginia Fisheries Laboratory in the summer of 1944, under the direction of Dr. Curtis L. Newcombe.

The first Pinnotherid larva to be described was the zoea of *Pinnotheres pisum* Leach, the British pea crab. This species has three or more zoeae. Other members of the family are said to have two, three or four zoeal stages; in some species a megalops stage has been described, while one at least is said to pass directly from the last zoea to the first crab. The family Pinnotheridae is noted for the great diversity of the zoeae in different species, and this lack of uniformity seems to apply also to the number of larval stages. Faxon (1879) stated that the last zoea of *Pinnixa chactopterana* molted directly into a first crab stage, without a megalops stage. Smith (1880) found a megalops in another species of *Pinnixa*. There are only two published descriptions of Pinnotherid megalops: *Pinnotheres veterum* by Lebour (1928) and *Pinnotheres taylori* by Hart (1935). In the latter there are two zoeae and one megalops.

The first zoea of *Pinnotheres ostreum* was hatched from the egg by Birge (1878), but he was unable to rear second zoeae. Hyman (1925) obtained the first zoeae of *Dissodactylus mactitac*, *Pinnotheres maculatus*, and *P. ostreum* by hatching, and described the second zoea of *P. ostreum* from specimens reared from first zoeae caught in plankton.

In the present study, all specimens were reared in the laboratory from the egg. *Pinnotheres ostreum* was found to have four zoeal stages and one megalops, followed by the first crab.

## METHODS

Ovigerous female crabs were removed from oysters and the egg strands were cut away and placed in shallow enamel pans containing sea water. The salinity of the water used varied from 20 to 26 parts per thousand while the temperature was approximately 23° C.

On July 15, 1944, a medium brown sponge was removed from an oyster crab and placed in water. One week later first zoeae emerged from the eggs. Ecdysis

<sup>1</sup> Contribution from the Virginia Fisheries Laboratory of the College of William and Mary and Commission of Fisheries of Virginia, Number 27.

of the larval cuticle occurred simultaneously with hatching. Five days later some first zoeae molted to become second zoeae, and three days afterward third zoeae appeared. All of these third zoeae died in ecdysis without becoming fourth zoeae.

A second experiment was begun on July 16, 1944. A younger sponge of bright orange color was placed in hatching pans. First zoeae hatched at the end of twelve days and after three days these specimens became second zoeae. In an additional five days the third instar appeared, and after seven more days several individuals reached the fourth zoeal stage. There was a high mortality rate during this ecdysis. The fourth zoeae were placed in individual glass finger bowls. Six days later one megalops completed a successful ecdysis and became a first crab. Three other fourth zoeae also reached the megalops stage and eventually one of these became a first crab, but the times were not noted. The total time in this experiment, from the recently laid orange eggs to the megalops stage, was 33 days, and the total time required to obtain the first crab was 38 days. The period of larval development, from hatching to first crab, was 25 days.

The length of time spent in each instar during the development seemed to depend on a number of factors, food abundance and water temperature having the most obvious effect. Few complete records on embryonic and larval development of crabs have been published. Hart (1935) found that four or five weeks were required for *Pinnotheres taylora* to pass through two zoeal instars and one megalops stage.

The food used in rearing *P. ostreum* larvae consisted of concentrated plankton from York River. A yellow dinoflagellate was eaten most readily by the zoeae, but they also fed on crab eggs. Excess food was removed after feeding to prevent fouling. Water was changed every second day and was aerated several times daily by pipetting.

In the morphological study of life history stages it was necessary to use the material conservatively, since very few specimens of each stage were preserved. Killing and preservation were in 5 per cent formalin. For study, each specimen was placed in a drop of glycerine on a slide, a cover glass was put on, and the specimen was drawn *in toto* without flattening. Megalops and first crab stages were placed on depression slides to avoid distortion. Cover glasses were removed after *toto* drawings had been made, and the specimens were dissected with ordinary dissecting needles. Appendages were pulled off, not cut. Dissected parts were arranged in the glycerine with needles and the cover glass replaced, with slight pressure. No great difficulty was experienced with this method; in nearly every case at least one member of each pair of appendages was found intact and in position to draw. All drawings were made with a camera lucida; only very fine details such as the setules on feathered setae were added free hand.

#### DESCRIPTION OF STAGES

##### *First zoea* (Figs. 1, 3, 4, 6, 8, 9, 12)

In living specimens, fully extended but not flattened, the total length is 0.90 mm. In formalin-preserved specimens the total length is 0.7 to 0.9 mm. The carapace averages 0.42 mm. in length and 0.27 mm. in width. The eyes are 0.11 to 0.13 mm. in diameter. There are no dorsal or lateral spines on the carapace.



The abdomen has five segments, not counting the telson; its width increases toward the posterior end. The second and third abdominal somite bear short triangular lateral knobs. The telson has three toothlike lobes, with three setae on each side between the lateral and median lobe. The chromatophores are distributed as described by Hyman (1925). The antennules are folded to a spherical form, and bear two large and one small aesthete or sensory hair. The antennae are not visible. The first maxilla (maxillule) has no "epipodital hair"; the protopodite bears a chromatophore; the proximal (coxopodite) lobe bears four setae, the distal (basipodite) lobe has five, and there are four setae arranged in two groups of two each on the two-segmented endopodite. The second maxilla has a chromatophore on the protopodite, five setae on the coxopodite, eight or nine on the basipodite, three on the endopodite, and four on the scaphognathite. The first maxilliped has chromatophores in the coxopodite and basipodite; the basipodite bears eight setae, the exopodite has four long terminal setae or "swimming hairs," and the five-segmented endopodite has one seta on the proximal segment, two on the second, one on the third, two on the fourth, and five on the terminal segment of which four are terminal. The second maxilliped has a chromatophore on the coxopodite, a chromatophore and four setae on the basipodite, four "swimming hairs" on the exopodite, and four setae on the terminal segment of the two-segmented endopodite. No buds of other appendages are visible.

*Second zoea* (Figs. 2, 10, 13).

The total length of preserved specimens is 1.48 mm., but these specimens have an abnormal swelling between the thorax and abdomen; the normal length would be somewhat less. The length of the carapace is 0.57 mm. The eye is 0.12 to 0.14 mm. in diameter. The first maxilla has an external seta or "epipodital hair," and there are five, seven, and four setae on the coxopodite, basipodite and endopodite, respectively. The second maxilla has six, nine, and three setae on these three endites; the scaphognathite has three apical setae and five near the basal end. The

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EXPANDED OF PLATE I

All drawings made with camera lucida from specimens killed and preserved in 5 per cent formalin, mounted in glycerine. Scale A represents 0.4 mm. in Figures 1, 2, 6, 7, 7a, and 8. Scale B represents 0.1 mm. in Figures 3 and 4.

FIGURE 1. First zoea of *Pinnotheres ostreum*, hatched from egg in laboratory, drawn with  $10 \times 10 \times$  lenses.

FIGURE 2. Second zoea of *P. ostreum* reared from egg in laboratory, drawn with  $10 \times 10 \times$  lenses.

FIGURE 3. First maxilliped of first zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses; swimming hairs of exopodite cut off to allow placement of figure on plate.

FIGURE 4. Second maxilliped of first zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses; swimming hairs cut off figure.

FIGURE 5. Telson of third zoea, *P. ostreum*, drawn with  $15 \times 10 \times$  lenses.

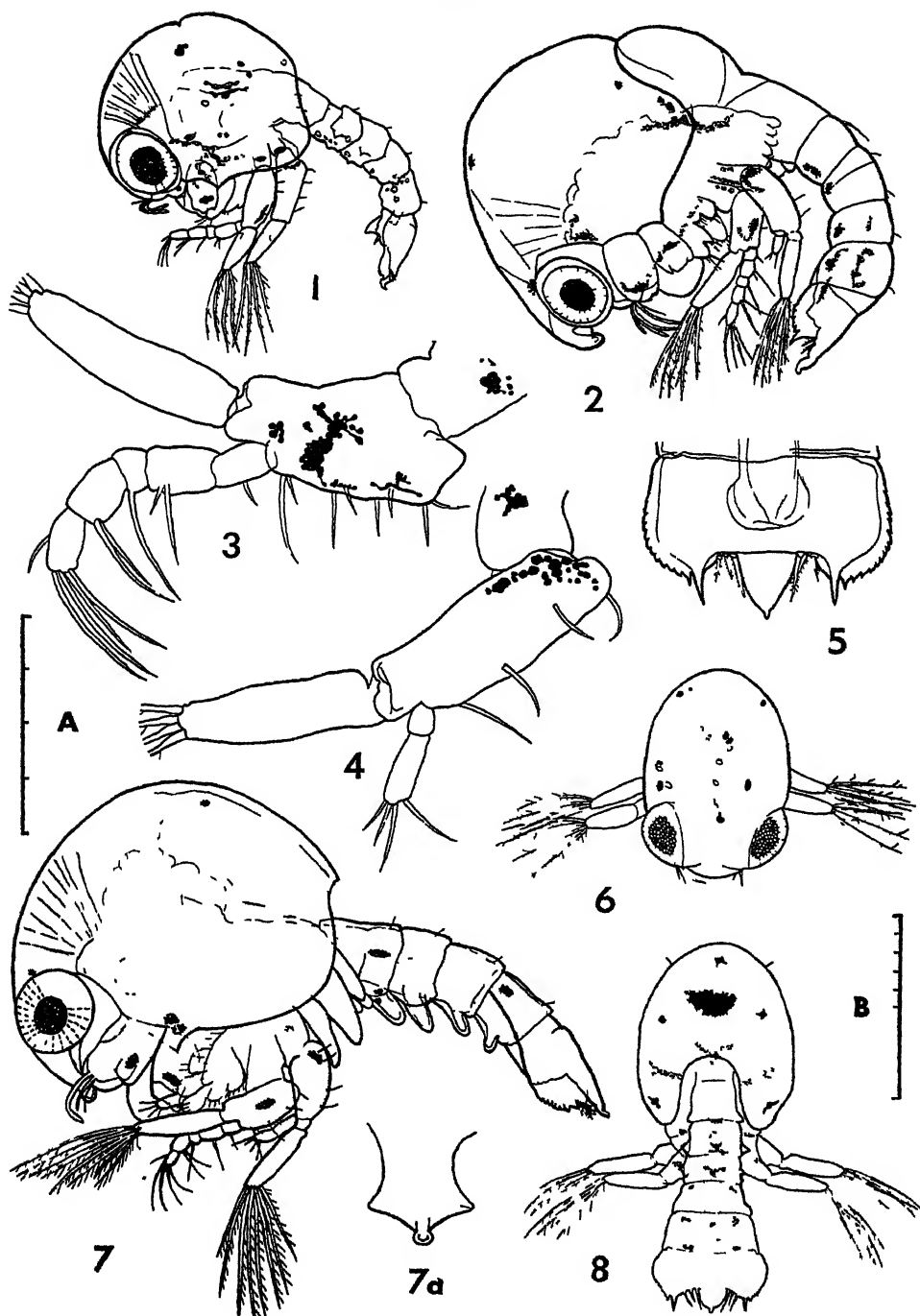
FIGURE 6. Anterior view of first zoea, *P. ostreum*, drawn with  $10 \times 10 \times$  lenses.

FIGURE 7. Third zoea of *P. ostreum* reared from egg in laboratory, drawn with  $10 \times 10 \times$  lenses.

FIGURE 7a. Rostrum of third zoea, *P. ostreum*, frontal view, drawn with  $10 \times 10 \times$  lenses.

FIGURE 8. Posterior view of first zoea, *P. ostreum*, showing dorsal view of abdomen, drawn with  $10 \times 10 \times$  lenses.

PLATE I



first and second maxillipeds each have six swimming hairs on the exopodite. Buds of other thoracic appendages can be seen, but there are still no buds on the abdomen. Other features are as in the first zoea.

*Third zoea* (Figures 5, 7, 7a, 11, 14)

The total length of preserved specimens is 1.3 mm. The carapace is 0.60 mm. long (0.58 to 0.63). The eye is 0.14 to 0.16 mm. in diameter. The antennule bears three large and one small aesthete. The first maxilla has five setae on the coxopodite, eight or nine on the basipodite, and four on the endopodite. The second maxilla has seven to nine setae on the coxopodite, nine or ten on the basipodite, and three on the endopodite; the scaphognathite has five apical setae and eight to thirteen setae between the proximal end and the apical setae. The coxopodite of the first maxilliped has three setae, the basipodite has eight or nine setae, and the exopodite has eight swimming hairs. The exopodite of the second maxilliped also has eight swimming hairs. Buds of the other thoracic appendages are prominent and buds of the abdominal appendages are visible. Other features are as in the first zoea, except for size.

*Fourth zoea*

Only four or five specimens were obtained, and they molted to become megalops before they could be studied. The only known feature is the possession of ten swimming hairs on each of the maxillipeds.

*Megalops* (Figs. 15, 16, 17, 18)

The total length is 1.0 to 1.05 mm. The carapace is 0.60 mm. long and 0.58 mm. wide. The abdomen, extended, is 0.40 to 0.45 mm. long and 0.17 mm. wide across the second segment. The eye is about 0.14 mm. in diameter. The carapace has no spines, on the rostrum or elsewhere, but has four to nine setae along each lateral edge. The fifth leg has no "feelers" on the last segment. The antennule has seven or eight aesthetes on the distal segment (outer flagellum). The antenna

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EXPLANATION OF PLATE II

All drawings made with camera lucida from specimens killed and preserved in 5 per cent formalin, mounted in glycerine. Scale A represents 0.4 mm. in Figures 15 and 18. Scale B represents 0.1 mm. in Figures 9, 10, 11, 12, 13, and 14. Scale C represents 0.1 mm. in Figures 16 and 17.

FIGURE 9. First maxilla of first zoea, *Pinnotheres ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

FIGURE 10. First maxilla of second zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

FIGURE 11. First maxilla of third zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

FIGURE 12. Second maxilla of first zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

FIGURE 13. Second maxilla of second zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

FIGURE 14. Second maxilla of third zoea, *P. ostreum*, drawn with  $7.5 \times 43 \times$  lenses.

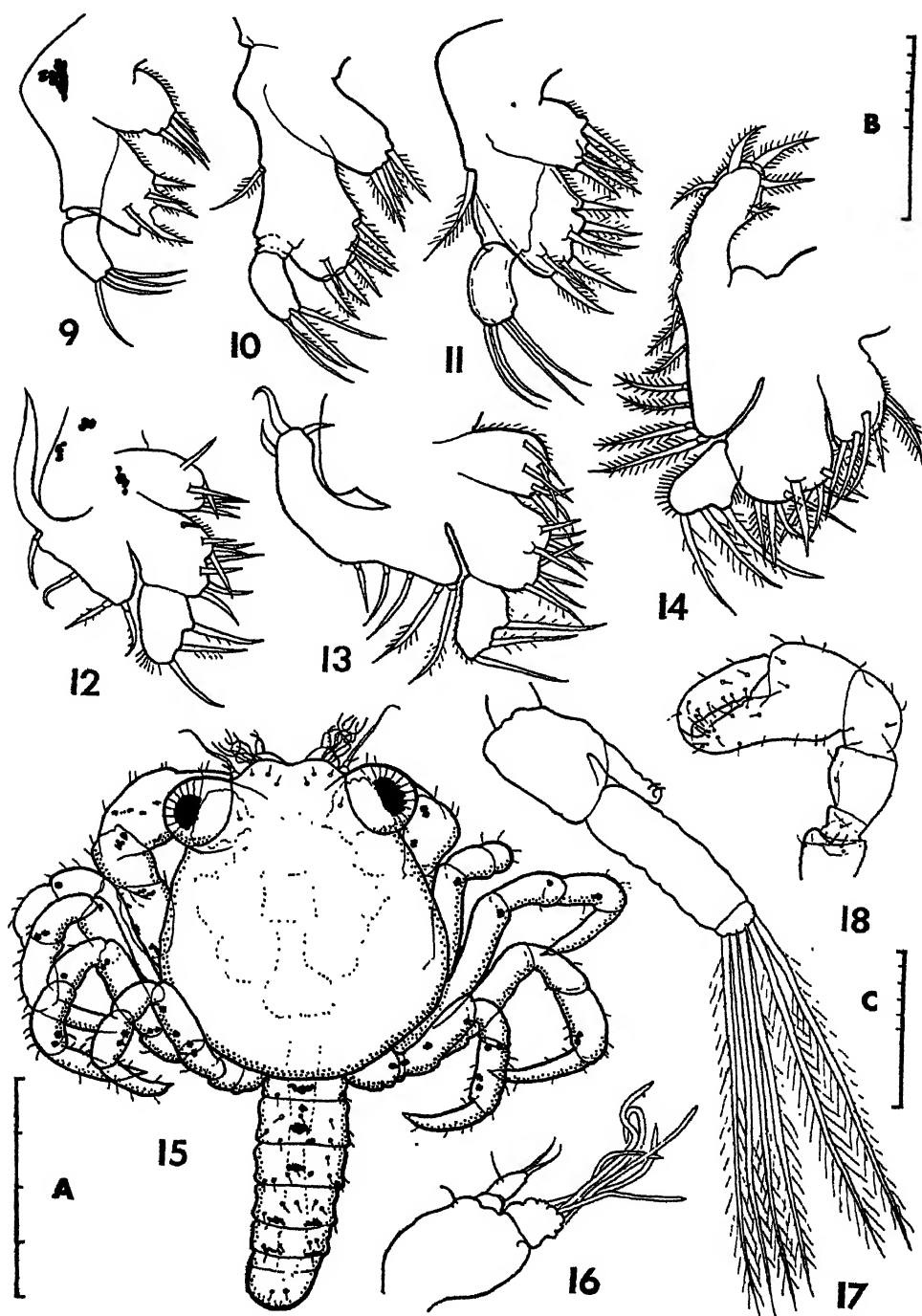
FIGURE 15. Megalops of *P. ostreum* reared from egg in laboratory, dorsal view, drawn with  $10 \times 10 \times$  lenses.

FIGURE 16. Distal portion of antennule of megalops, *P. ostreum*, drawn with  $6 \times 44 \times$  lenses.

FIGURE 17. First pleopod of megalops, *P. ostreum*, drawn with  $6 \times 44 \times$  lenses.

FIGURE 18. Dorsal view of right cheliped of megalops, *P. ostreum*, drawn with  $10 \times 10 \times$  lenses.

PLATE II



is five-segmented with a long slender process and a short seta on the distal segment. The abdomen seems to have six segments, but the sixth segment is very indistinct and may be fused with the telson. There are four pairs of pleopods, on the second, third, fourth and fifth segments; the exopods of the first three pairs bear six swimming hairs; those of the last pair have only five. (In the other specimen, the first pleopods have five hairs, the second and third have six, and the fourth have four.)

*First crab* (Figs. 19, 20, 21, 22, 23)

The carapace is 0.61 mm. long and 0.59 mm. wide. The diameter of the eye is about 0.14 mm. There are no spines on the carapace, but each lateral edge bears seven to nine setae. The last two segments of the third and fourth legs bear long plumose setae, but there are no "feelers" on the fifth leg.

It should be noted that these two laboratory-reared specimens are considerably smaller than the "first stage females" of Stauber (1945). Stauber used this name for the youngest females which invade the oyster, evidently not intending to imply that they were in the first crab instar. Probably there are two or more free-living crab instars before the invasive stage. The smallest "first stage females" found in oysters by Stauber were 1.4 mm. wide, and the smallest males were 1.5 mm.

*Oyster crabs in Virginia oysters*

No systematic study of the distribution, abundance, and effects of oyster crabs in Virginia has been made, but field notes accumulated by the Virginia Fisheries Laboratory give some data on these points. *Pinnotheres ostreum* has been found on all Virginia oyster grounds which have been observed. The percentage of oysters infested, on different oyster rocks, varies from less than 1 per cent to over 80 per cent, averaging around 30 or 40 per cent. Immature and mature female oyster crabs are found singly in Virginia oysters throughout the year. In only 5 of 276 infested oysters examined in 1943-44 were two crabs found in one oyster. Multiple infestations by male and female early-stage crabs, such as were described in New Jersey by Stauber (1942, 1945), were found only once in Virginia, at Cedar Island, James River, in the summer of 1945. Notes on females bearing sponges extend only from June through August, but the actual spawning season is probably much longer than this. *P. ostreum* zoeae have been found in plankton tows from June through August, but few tows were made in other months.

Significant damage to the gills of oysters by oyster crabs has been noted in many cases. Field notes on the condition of oysters generally show poorer condition in

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EXPLANATION OF PLATE III

All drawings made with camera lucida from specimens killed and preserved in 5 per cent formalin, mounted in glycerine. Scale A represents 0.4 mm. in Figures 20, 22, and 23. Scale C represents 0.1 mm. in Figures 19 and 21.

FIGURE 19. Antenna of first crab instar, *P. ostreum*, drawn with  $6 \times 44 \times$  lenses.

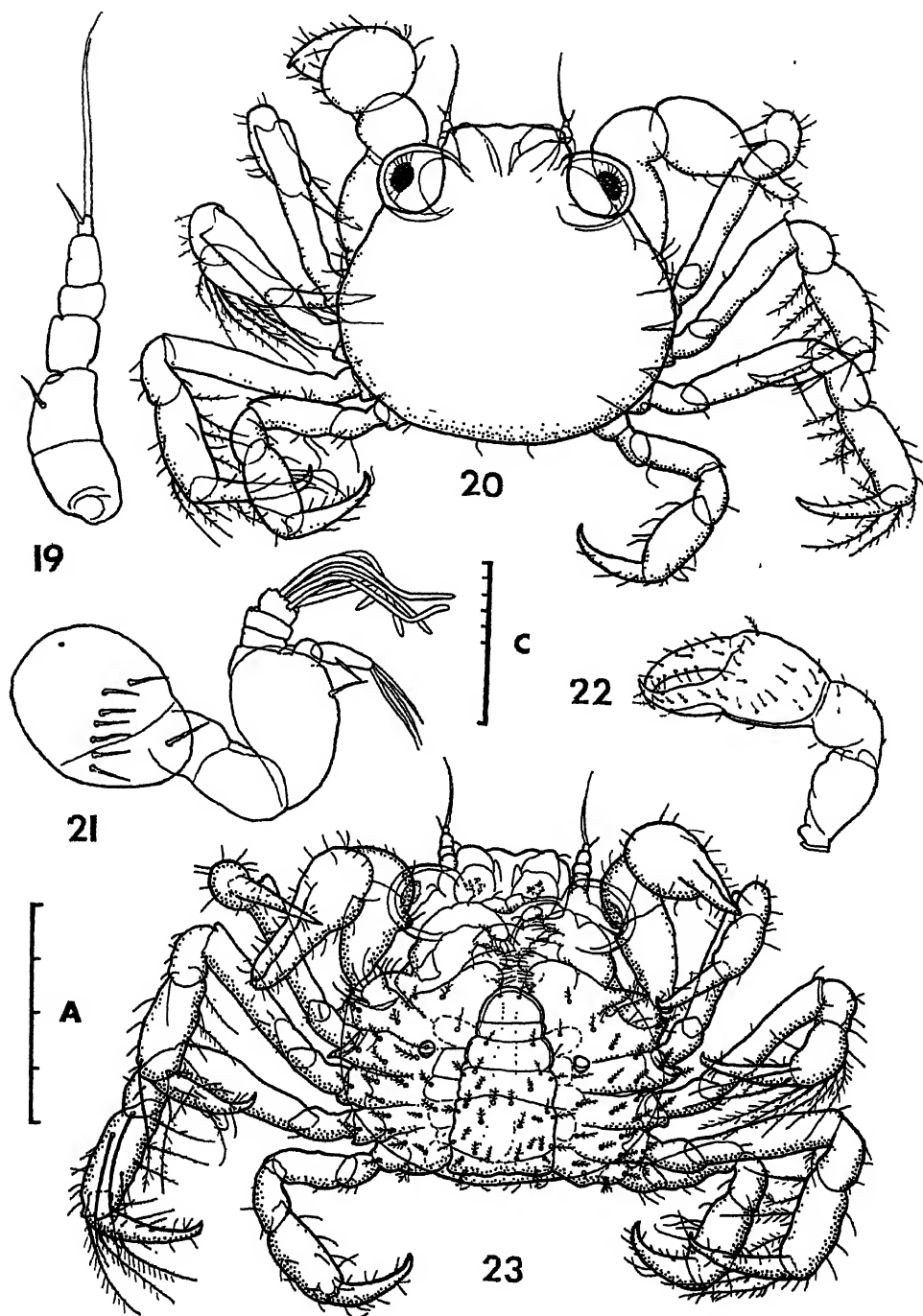
FIGURE 20. First crab instar, *P. ostreum*, reared in laboratory, dorsal view, pigment omitted, drawn with  $10 \times 10 \times$  lenses.

FIGURE 21. Antennule of first crab instar, *P. ostreum*, drawn with  $6 \times 44 \times$  lenses.

FIGURE 22. Right cheliped of first crab instar, *P. ostreum*, dorsal view, drawn with  $10 \times 10 \times$  lenses.

FIGURE 23. First crab instar of *P. ostreum* reared from egg in laboratory, ventral view, drawn with  $10 \times 10 \times$  lenses.

PLATE III



crab-infested than in uninfested oysters, but there are often some crab-infested oysters which show excellent condition. Miss Alice Elizabeth Overcash, in an unpublished thesis (1946) submitted to the College of William and Mary, has studied the "index of condition" of Virginia oysters, including many with oyster crabs. She reports that this index  $\frac{(1000 \times \text{dry weight of meat in grams})}{(\text{Volume of shell cavity in milliliters})}$  averaged only 82.3 for crab-infested oysters in York River, compared to 90.6 for oysters without crabs. In Rappahannock River she found the mean index of oysters with crabs to be only 71.7, while the entire sample averaged 90.0. Both samples showed significantly poorer condition in infested oysters.

There seems to be no doubt that oyster crabs do injure oysters to some extent, but we have no evidence that they have ever caused the death of oysters in Virginia, as they did in the New Jersey outbreak reported by Stauber (1942, 1945).

#### SUMMARY

1. The early stages of *Pinnotheres ostreum* have been reared in the laboratory from the egg to the first crab.
2. There are four zoeal stages and one megalops stage. The four zoeal instars are distinguished by having 4, 6, 8, and 10 swimming hairs on the exopodites of both maxillipeds.
3. The time required for development from the early egg (from orange-colored egg mass) to the first crab instar was 38 days. From hatching to the first crab required 25 days.
4. The first crab being only 0.6 mm. wide, at least one more instar must intervene before *P. ostreum* reaches the "first stage" described by Stauber as the youngest crabs which invade the oyster.
5. Oyster crabs are widely distributed and abundant in Virginia waters. They tend to keep oyster meats in relatively poor condition, but have never been observed to cause mortality of oysters in Virginia.

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# ELECTROKINETIC STUDIES OF MARINE OVA. V. EFFECT OF PH-CHANGES ON THE SURFACE POTENTIALS OF SEA-URCHIN EGGS

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In a preliminary account (Dan, 1931), it was reported that the cataphoretic potentials of the unfertilized eggs of the sea-urchin, *Arbacia punctulata*, could not be reversed in sign on raising the concentration of hydrogen ions of the surrounding medium. The present paper reports experiments of a similar type which were repeated on other kinds of sea-urchins, *Anthodidaris crassispina* and *Pseudocentrotus depressus*, and the study was further extended to include fertilized eggs.

## METHOD

*Removal of the jelly.* When the unfertilized surface was studied, unless otherwise stated, the jelly was removed by acid. While a dense suspension of eggs was being gently shaken, 1/10 N HCl was added drop by drop until the eggs began to show the first sign of sticking to the glass container. After a few more gentle shakings, a large quantity of sea water was added and the eggs were washed thoroughly by decantation. By this means, it was possible to reduce the time of the eggs' coming in contact with the concentrated acid to the minimum (less than 10 seconds).

*pH adjustment.* HCl was added to filtered sea water to an amount ample to break down the bicarbonate buffer system. After equilibration, more acid or alkali was added to adjust the pH value to the desired level by indicators. When the desired pH levels were near neutrality, 1/10 N acid or alkali was used, but when they were far removed from neutrality, 1/2 N solutions were added to minimize the lowering of the osmotic pressures. A slight lowering of the osmotic pressure by using 1/10 N within the limit here encountered does not affect the surface potentials of sea-urchin eggs (Dan, 1936). During the experimentation, the supernatant solution of the egg suspension was frequently checked for pH value to insure its constancy.

In very acidic media, the eggs were injured. At pH 3.5 or 4.0 they could still keep a fairly high fertilizability, if they were left unstirred in the acid and later fertilized after being returned to the normal medium. However, in the present experiment, stirring was technically unavoidable to some extent. In spite of this, pH values down to 2.0 were used for the purpose of obtaining a sufficient insight into the electrokinetic property of the egg surface, taking advantage of the fact that the killing of the eggs does not modify the potentials (Dan, 1934; 1936; see also Winslow, Falk and Caulfield, 1923; Abramson, 1929).



*Measurement of the potential.* The method of measuring the cataphoretic potentials was practically the same as was reported in the preliminary note and more fully discussed in the first paper of this series (Dan, 1933). This was to use a horizontal microscope on which a Northrop-Kunitz chamber was mounted. Since the stage of the horizontal microscope was situated vertically, the short axis of the cataphoretic chamber came to lie vertically. The chamber was filled with an egg suspension, and when the eggs began to settle, their paths of fall were recorded by a camera lucida. The focus of the microscope was previously adjusted to the level at  $\frac{1}{2}$  of the depth of the chamber (Smoluchowski's layer) and only the eggs which came into sharp focus were selected for measurement. Two points were recorded to determine the path of fall; the electric current was then sent horizontally (i.e., at right angles to the path of fall) and the new path was traced. The deviation of the new path from the extrapolation of the initial path (with no current) corresponds to the cataphoretic movement. The interval of observation was measured by counting the beats of a metronome which was beating once in half a second. The potential drop within the chamber was measured by platinum poles inserted into the chamber through the glass wall by using a voltmeter with a high internal resistance (10,000 ohms, which is about 10 times the resistance of the chamber filled with sea water). The cataphoretic potentials were calculated by the formula  $u = DH\zeta/4\pi\eta$ , assuming that the dielectric constant of sea water is 80.

In order to minimize daily fluctuations in technique, readings were, as a rule, taken in three adjacent pH values on one day and different combinations of three pH's were studied each day. The data were later handled collectively according to the pH values.

#### ANTHOCIDARIS CRASSISPINA

The question, whether or not the surface potential is affected by fertilization, has been taken up by several investigators. Gray (1916) examined the agglutination of unfertilized and fertilized eggs of *Sphaerechinus* by  $\text{CeCl}_3$  and came to the conclusion that fertilized eggs are carrying a more negative potential than unfertilized eggs, basing his conclusion on the fact that a more concentrated solution of cerium cations was required to agglutinate fertilized eggs than unfertilized ones. Runnström's observation (1929, p. 229) tends to support this conclusion. Vlès and Nouel in sea-urchin eggs (1922) and Fauré-Fremiet in the egg of *Sabellaria alveolata* (1924, p. 291) made observations on the behavior of these eggs in an electric field and arrived at the diametrically opposite conclusion, while Szent-Györgyi (1921) contended that sea-urchin eggs bore no charge.

However, some of the above investigations are based on indirect evidence, while the others are not quite free from technical dangers. Therefore, it was considered worth while to re-examine this point by a more direct technique. As a result, in the present paper, not only were the potentials on unfertilized and fertilized eggs compared in normal sea water, but the pH-potential curves of both were constructed through a wider range of the pH-scale. *Anthocidaris crassispina* was selected as one material. In the measurements of the fertilized eggs, the fertilization membrane was left intact. The perivitelline space of this species is very small, which is technically advantageous.

Data are summarized in Table I and graphically represented in Figure 1. (1) The potentials on both unfertilized and fertilized eggs of *Anthocidaris* cannot be

reversed in sign as far down as pH 2.0. (2) The maxima of both curves are found in the neighborhood of pH 7.0. (3) Though a statistical separation of the potentials on unfertilized and fertilized eggs at corresponding pH's is impossible, because of the wide fluctuation of the readings, the fact that the average values of the potentials on the fertilized eggs are invariably more positive than those of the unfertilized can be taken as a strong indication that the former are really more

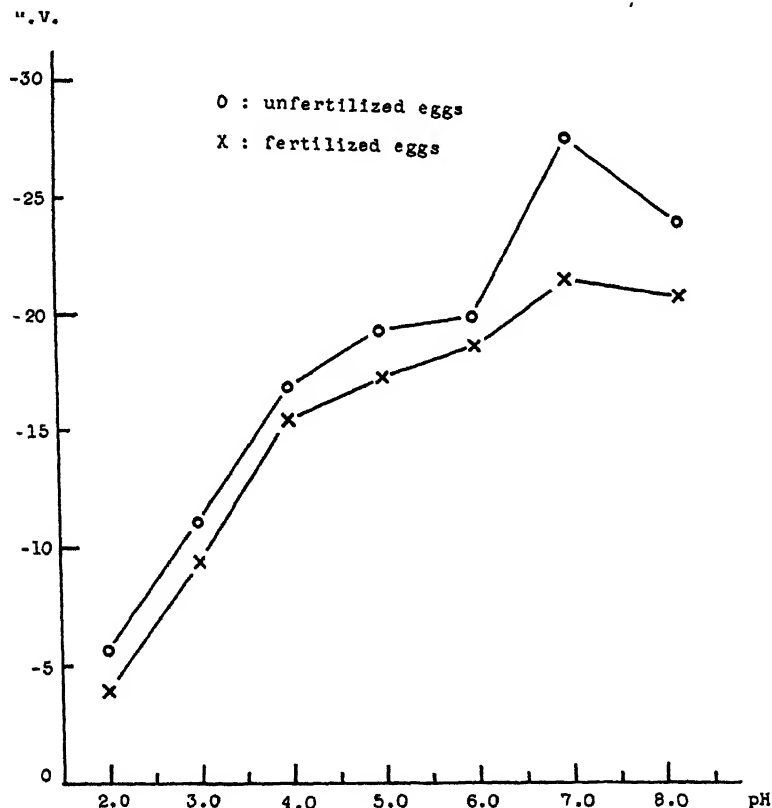


FIGURE 1. *Anthocidaris crassispina*.

positive. Needless to say, this is not due to an increase in the viscosity of the medium by the addition of the seminal fluid, since the eggs are later washed thoroughly. (4) The pH-potential curve for the unfertilized eggs is more zig-zag than that for the fertilized eggs, especially between pH 6 and 7. A repetition of the measurement in the following season (Series II of Table I) gave similar results which were incorporated in the final calculation.

#### PSEUDOCENTROTUS DEPRESSUS

In order to test the generality of the *Anthocidaris* findings, another sea-urchin *Pseudocentrotus depressus* was studied. However, in this case, attention was paid

TABLE I

*Potentials on the egg surface of Anthocardis crassispina in millivolts with standard errors*

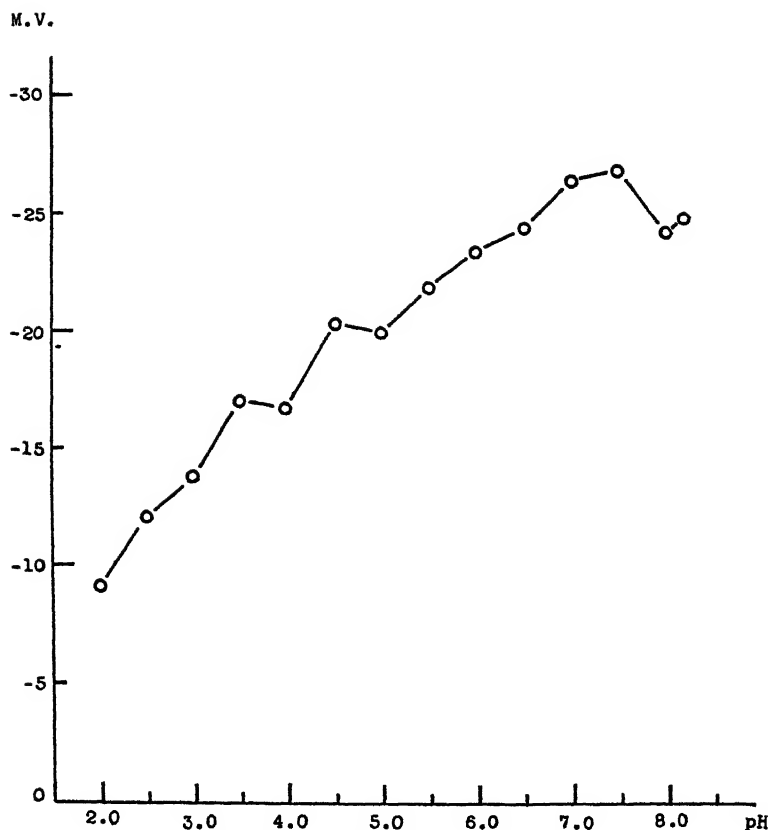
Medium	Unfertilized eggs without jelly			Fertilized eggs
	Series I	Series II	I and II combined	
Sea water	$-24.9 \pm 0.78$	$-23.2 \pm 0.38$	$-23.9 \pm 0.34$	$-20.8 \pm 0.79$
pH 7	$-28.5 \pm 0.62$	$-26.3 \pm 0.57$	$-27.4 \pm 0.46$	$-21.5 \pm 0.58$
pH 6	$-19.9 \pm 0.71$	$-19.7 \pm 0.45$	$-19.9 \pm 0.35$	$-18.6 \pm 0.46$
pH 5	$-19.9 \pm 0.71$	$-18.5 \pm 0.43$	$-19.3 \pm 0.38$	$-17.2 \pm 0.61$
pH 4	$-13.3 \pm 0.62$	$-16.8 \pm 0.39$		$-15.5 \pm 0.47$
pH 3	$-11.0 \pm 0.59$			$-9.4 \pm 0.48$
pH 2	$-5.6 \pm 0.90$			$-4.0 \pm 0.81$
	Unfertilized eggs with jelly			
Sea water	$-24.1 \pm 0.41$			

chiefly to the unfertilized surface. pH intervals were taken to apparent 0.5 units of the indicators used. \*Data are summarized in Table II and Figure 2. As is evident from the figure, the general trend of the curve is very much the same as that of the two previous ones, with no reversal of the sign even in the most acidic medium (pH 2) and with a maximum slightly on the alkaline side of neutrality.

TABLE II

*Potentials on the egg surface of Pseudocentrotus depressus in millivolts with standard errors*  
Unfertilized eggs without jelly

Medium	Potentials	Indicators
Sea water	$-25.0 \pm 0.62$	Phenol red
pH 8.0	$-24.4 \pm 0.55$	
pH 7.5	$-27.0 \pm 0.69$	
pH 7.0	$-26.6 \pm 0.57$	
pH 6.5	$-24.5 \pm 0.53$	Brom cresol purple
pH 6.0	$-23.6 \pm 0.60$	
pH 5.5	$-22.0 \pm 0.44$	
pH 5.0	$-20.1 \pm 0.53$	Brom cresol green
pH 4.5	$-20.4 \pm 0.46$	
pH 4.0	$-16.8 \pm 0.43$	Brom phenol blue
pH 3.5	$-17.0 \pm 0.47$	
pH 3.0	$-13.8 \pm 0.55$	
pH 2.5	$-12.1 \pm 0.40$	Meta cresol purple
pH 2.0	$-9.1 \pm 0.59$	
Fertilized eggs with jelly		
Sea water	$-28.5 \pm 0.71$	

FIGURE 2. *Pseudocentrotus depressus*.

## DISCUSSION

In the first paper of this series, it was reported that the potentials on the unfertilized and fertilized (with the fertilization membrane) eggs of *Arbacia punctulata* were  $-30.3$  and  $-28.7$  millivolts respectively and the potential on the unfertilized eggs in a suspension of dead sperm was  $-26.7$  millivolts. Comparing these figures with the sperm potential and its change under the influence of the egg water of the same species studied by Mudd, Mudd and Keltch (1929): the potential they found on the sperm was  $-22.0$  millivolts in sea water and "an increase in negative charge by 13 per cent followed on addition of the egg water." This means that the potential on the sperm in the egg water is  $-24.9$  millivolts. Now Mudd's experiment and the author's are reciprocal to each other. While the sperm potential which is  $-22.0$  millivolts changes to  $-24.9$  millivolts in the presence of the egg proteins, the egg potential which is  $-30.3$  millivolts at the beginning changes to  $-26.7$  millivolts in the presence of the sperm proteins. It is highly probable that the mixing of two kinds of proteins will shift the resultant potential somewhere in between the two extremities. This is particularly likely because both the seminal fluid in the author's experiment and the egg water used by Mudd et al. are ex-

tremely low in concentration. If a protein is added in high concentration, the potential will coincide with that of a pure protein.

The above postulate acquires a stronger support when the potential changes of other forms are considered. Mudd et al. studied not only the sperm potential of the sea-urchin (*Arbacia punctulata*) but also those of a starfish (*Asterias forbesii*) and a sand-dollar (*Echinarachnuiis parma*) and further investigated their changes in egg waters in various combinations. Fortunately, the potentials of the eggs of these species have been measured by the author (Dan, 1934). As is shown in Table III, a sperm potential in egg water either falls between the potentials of the pure sperm and of the egg or coincides with one of them within 1 millivolt. In Mudd's data, the fact stands out as peculiar that the sea-urchin egg water has a specific power to cause a striking increase in the negative potential of any kind of sperm (see Table III, column 3) but this is now easily understandable because the egg protein of *Arbacia* has an exceptionally high negativity.

TABLE III

Comparison of egg potentials, sperm potentials and sperm potentials in egg water. As egg potential, the potential on unfertilized eggs without jelly is used. The figures in parentheses are the percentage changes in negativity of sperm potentials under the influence of egg waters.

Sperm potential \ Egg potential	Echinarachnius ca. -20.0 m.v.	Arbacia -30.3 m.v.	Asterias -19.9 m.v.
Echinarachnius -16.3 m.v.	-19.6 m.v. (+20%)	-21.7 m.v. (+33%)	-16.3 m.v. ( 0%)
Arbacia -22.0 m.v.	-21.6 m.v. (- 2%)	-24.9 m.v. (+13%)	-18.9 m.v. (-14%)
Asterias -18.1 m.v.	-17.7 m.v. (- 2%)	-21.7 m.v. (+20%)	-19.2 m.v. (+ 6%)

The present experiment was started with the above view in mind and it was hoped that the difference in the natures of the two proteins (if they are proteins) might be brought up more clearly by constructing pH-potential curves in a wider range. As a result, in the *Anthocidaris*-series, after fertilizing the eggs by the usual technique, excess of sperm fluid was added to secure a complete adsorption and then the eggs were thoroughly washed free from seminal fluid. In spite of this, both curves followed very similar courses except for the fact that the curve for the fertilized eggs consistently stayed below that of the unfertilized eggs.

It may be of some significance to point out in this connection that cholesterol has a very high electric mobility among substances investigated so far. According to Sugawara (1943a, 1943b) the fertilization membrane and its precursor of sea-urchin eggs are dissolved by proteolytic enzymes. This does not necessarily mean that the fertilization membrane and its precursor are made up solely of proteins. They are probably a compound of proteins and lipids and it is not impossible to imagine that one of the constituents predominates in the cataphoretic behavior. Further elucidation is greatly desired.

Concerning the course of the pH-potential curves, the absence of the isoelectric point and the existence of a maximum in the vicinity of pH 7 are worth noticing. Even though nothing definite can be based on the data at hand, these two points may be more or less connected with the high salt concentration of the medium, because cases are known in which the addition of salts either shifts the isoelectric point to the more acid side (Linderstrøm-Lang and Kodama, 1925; Sørensen and Sladek, 1929; Fauré-Fremiet and Nichita, 1927; Haffner, 1922) or brings the maximum around neutrality (Winslow, Falk and Caulfield, 1923). The absence of the isoelectric point here observed is contrary to Ashbel's finding (1931). Her paper states little about the technique of measurement. As far as this author's result is concerned, the pH-potential curves of sea-urchin eggs run quite close to those of *Bacillus cereus* in change of hydrogen ion concentration in concentrated salt solutions studied by Winslow, Falk and Caulfield (1923). The curves resemble each other not only in having no reversal of the sign in the acid range and having maxima near pH 7, but also in a decrease in the migration velocity (or the potential) in the alkaline region. This third similarity will be discussed briefly.

In the above-cited investigation of Winslow, Falk and Caulfield, *Bacillus cereus* loses its negative charge at about pH 10. In *Bacillus cereus* and *Bacillus coli*, the signs of the zeta potentials are reversed around pH 13.5 (Winslow and Shaughnessy, 1924). In the present data on sea-urchin eggs, since the pH scale is not extended so far into the alkaline region, only a downward trend is suspected. On the other hand, however, there is a great deal of indirect evidence from agglutination experiments, indicating that the absolute magnitude of the surface potential of sea-urchin eggs does decrease in the extremely alkaline region. Gray (1916) and Vlès (1924) concordantly found that the eggs agglutinate in a high pH region.

Naturally, a critical analysis is required before we can conclude that in sea-urchin eggs agglutination is really an indication that the absolute magnitude of the potential has decreased as it is in the case of bacteria or blood cells. This problem will be taken up in a later paper.

#### SUMMARY

1. No isoelectric points were found in unfertilized eggs of *Pseudocentrotus depressus* and in both unfertilized and fertilized eggs (with fertilization membrane) of *Anthocardia crassispina* when the pH was changed down to 2 in sea water.
2. In all the above cases, the maxima are found in the neighborhood of neutrality. Consequently, in natural sea water (pH, 8.2), the absolute magnitude of the potential is below the highest level.
3. Fertilized eggs carry a less negative potential than unfertilized eggs.
4. The data are discussed in connection with the sperm potentials and their changes in egg water reported by Mudd et al.

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# ELECTROKINETIC STUDIES OF MARINE OVA. VI. THE EFFECT OF SALTS ON THE ZETA POTENTIAL OF THE EGGS OF *STRONGYLOCENTROTUS PULCHERRIMUS*

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In the third paper of this series (Dan, 1936a), the zeta potential of the eggs of *Arbacia punctulata* was measured in NaCl and CaCl<sub>2</sub>, both isotonic to sea water, and also in mixtures of the two in various ratios. From that study, the conclusion was drawn that the unfertilized surface of the *Arbacia* egg is ordinarily covered by a calcium compound of some sort which, however, dissolves away in the absence of calcium ions. This was based on three facts. (1) In higher calcium concentrations (more than 1/100 M of Ca in the isotonic mixture), the negative potential decreases, roughly corresponding to the calcium concentration, and this change is reversible. This is typical of the ionic effect of divalent cations and it shows that, within this range, the egg surface remains inert. (2) On the contrary, in lower calcium concentrations (less than 1/1000 M Ca), instead of the increase in negativity which is to be expected from the standpoint of the ionic effect, it decreases again and this decrease is irreversible. This is interpreted as due to the permanent loss of the calcium compound. (3) When *Arbacia* eggs are fragmented by centrifugal force into a lighter and a heavier part, the lighter part acquires a potential characteristic of a surface devoid of the calcium compound while the heavier part has a potential identical with that of the compound. This shows that the calcium compound forms a discrete membrane which ruptures on the fragmentation of the cell and is ultimately thrown down toward the heavy pole (Dan, 1936b).

In the first part of the present paper, the same sort of experiment is repeated with the present material and in the second part, the effect of cerium chloride is examined.

## MATERIAL AND METHOD

Eggs of the sea-urchin, *Strongylocentrotus pulcherrimus*, were used throughout. Unless specified, the jelly was always removed before the cataphoretic measurements. The method of removing the jelly and the method of measuring the zeta potential are the same as were used in the preceding paper (Dan, 1947).

## SODIUM-CALCIUM MIXTURES

The data are given in Table 1 and Figure 1. In the region of higher calcium concentrations, as in the case of *Arbacia*, the absolute magnitude of the negative potential decreases as the divalent calcium ions increase and this change in potential is perfectly reversible. The parallelism between the *Strongylocentrotus*-curve and the *Arbacia*-curve in this region is particularly striking (see Fig. 1). This means



that the potential change under consideration is a typical ionic effect due to calcium ions. On the contrary, the two curves diverge in the region of lower calcium concentrations. While the *Arbacia*-curve comes down in dilutions beyond 1/1000 M calcium in mixture, the *Strongylocentrotus*-curve goes sharply up, and this change is reversible.

TABLE 1

*The zeta potentials of the unfertilized eggs of Strongylocentrotus pulcherrimus under various conditions. The values are given in millivolts with standard errors.*

With jelly	-37.3±0.70
Without jelly	-33.9±0.50
Without jelly, 4 hours in NaCl, measured in sea water	-33.9±0.53
Jelly not removed, 3 hours in urea, measured in sea water	-32.9±0.60
Jelly removed by acid, 4 hours in urea, measured in sea water	-33.0±0.50
Heat-killed in NaCl	-26.8±0.44
1/2 M NaCl (intact cells)	-39.7±0.67
NaCl + 1/100 M CaCl <sub>2</sub>	-33.4±0.64
NaCl + 1/10 M CaCl <sub>2</sub>	-28.9±0.63
NaCl + 1/5 M CaCl <sub>2</sub>	-22.7±0.67
1/3 M CaCl <sub>2</sub>	-17.5±0.40

If the line of argument used for the case of *Arbacia* eggs is pursued further, the above result must be interpreted as indicating that either the *Strongylocentrotus* eggs do not react with calcium ions or the covering layer of the calcium compound of this species is not dissolved away in pure NaCl. The author inclines to adopt the latter view. To some extent, this is supported by the fact that *Strongylocentrotus* eggs are more resistant to cytolysis in NaCl than other species. Of course, this result should not be taken to mean that the covering layer of *Strongylocentrotus* eggs cannot be dissolved at all under any circumstance. The peptization of this compound may occur in some other solutions or even in 1/2 M NaCl if a different treatment is used. In the present experiment, for the change of medium, the following procedure was selected as standard. To 20 cc. of the egg suspension in a beaker, 200 cc. of 1/2 M NaCl was gently added and the suspension gently stirred to insure complete mixing. After the eggs had settled, as much as possible of the supernatant solution was siphoned off and fresh NaCl solution was gently added and stirred as before. This washing process was repeated from four to five times. Consequently, the eggs were in the NaCl solution for 30 to 40 minutes before measurement began.

In order to test other possibilities, in one series the time factor was changed. After the usual washings, the eggs were left in NaCl so that the total sojourn of the eggs in this solution was 4 hours. In spite of this the potential did not change (Table 1). In another series, isotonic urea solution was tried and this series was further divided into two groups. In one group, unfertilized eggs with jelly were put directly into urea and left there for 3 hours. Needless to say, the jelly was dissolved by the time cataphoretic measurement was begun. In the second group, the jelly was removed by acid and the eggs were left in urea for 4 hours. Still, no change was observed in the potential. The potential was found to change only after the eggs were killed by heat in NaCl. This is undoubtedly due to the effect of the internal granules (see Dan, 1933). Therefore the above result shows that

the surface calcium compound of *Strongylocentrotus* (if there is such a compound) is much more stable than that of *Arbacia* eggs. Unfortunately the number of washings was not changed. At any rate, a definite conclusion can be drawn that the egg surface of this species is quite resistant to the absence of calcium ions and, under the experimental conditions here dealt with, it can be looked upon as an inert surface through the entire range of calcium concentrations.

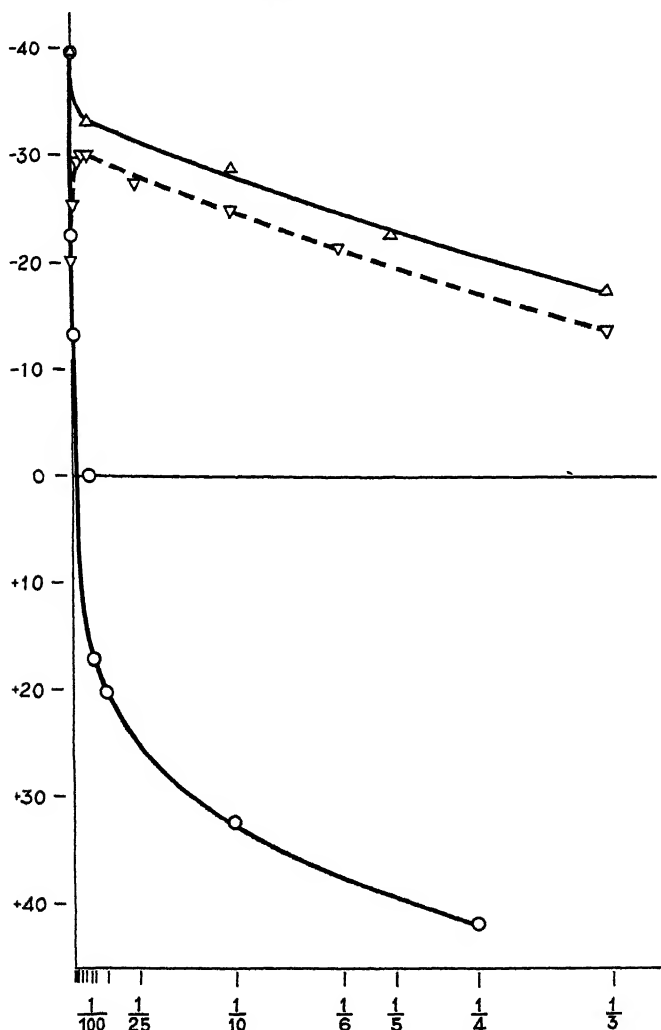


FIGURE 1. The change in the zeta potentials of the unfertilized eggs of *Strongylocentrotus* in mixtures of isotonic NaCl and CaCl<sub>2</sub> (Δ-Δ) and of NaCl and CeCl<sub>4</sub> (○-○). The ratios of the mixtures are expressed by the Ca- and Ce-concentrations respectively which are taken as the abscissae. The ordinates are the potential values. The broken line is the curve for *Arbacia* eggs in Na-Ca mixtures (▽-▽). Note the parallelism between the *Arbacia* curve and the *Strongylocentrotus* curve in higher Ca-concentrations and their divergence in the region of low Ca content.

## SODIUM-CERIUM MIXTURE

After examining the effect of calcium, cerium was studied as a representative of the polyvalent ions. The egg jelly was removed and the solutions were changed in the same way as in the calcium series. One-half M NaCl and  $\frac{1}{4}$  M  $\text{CeCl}_3$  were used either singly or in mixtures. For this purpose, the fact that the surface of *Strongylocentrotus* eggs remains unaffected in pure NaCl is of a great advantage, because it is impossible to mix the cerium solution with sea water on account of the formation of precipitates.

As far as the present material is concerned, the immersion of the eggs in cerium solution is not very harmful as long as the time of sojourn is not too long. On insemination of such eggs after having been returned to sea water, they are fertilizable except for the fact that the fertilization membrane fails to separate from the egg surface (tight membrane eggs).

On the other hand, there are two complications of a physicochemical nature involved in the use of cerium salts. One is the contamination of ceric ions in a preparation of cerous salt. Trivalent cerous ion is rather susceptible to oxidation and it spontaneously changes into tetravalent ceric ion. The former is colorless but the latter is light brown, and this transformation is clearly perceptible if the cerous solution is left in a transparent glass bottle. Unfortunately, this change seems to occur even in the crystals of the former as was revealed by a slight brownish tint of the cerous chloride crystals used by the author. But in the current study, no attempt was made either to obtain colorless crystals or to remove ceric ions, since it was considered that the maintenance of a constant proportion of the two ions would be sufficient for the present purpose. However, an attempt was made to know the ratio of the two by titration after Knorre's method (Knorre, 1897). Ceric ions, 0.0007 M, were found in 0.25 M solution by weight of the crystals; i.e., the ratio was 1:357. As a result, in the following paragraphs, the rather loose term "cerium solution" is used instead of specifying the condition precisely.

TABLE 2

*The hydrogen ion concentrations of various mixtures of 1/2 M NaCl and 1/4 M  $\text{CeCl}_3$ .  
The ratio of a mixture is expressed in Ce concentration after mixing.*

Concentrations (M) of Ce in NaCl	1/3000	1/1000	1/100	1/80	1/50	1/4
pH	7.0	6.8	6.2	5.7	5.6	4.7

The other complication is the change in hydrogen ion concentration of cerium solutions. As is well known, among polyvalent cations, it is very difficult to prepare solutions of different concentrations with the same pH, because the ions precipitate. In spite of this, measurements were carried out for a reason which will be explained below. The pH's of the cerium solutions used are given in Table 2.

## RESULTS

The result of cataphoretic measurements is given in Table 3 and graphically represented in Figure 1. As is evident, the curve is again typical of an adsorption isotherm, except for the potential value in  $\frac{1}{100}$  M cerium which is obviously off.

In this connection, it must be pointed out that sea-urchin eggs, like any colloidal particles, stick together when the surface potential is abolished. In a  $\frac{1}{100}$  M solution the eggs are extremely adhesive. (This point is specially investigated in a following paper.) On account of this, it is quite difficult to take accurate readings. As mentioned above, many eggs clump together in this medium and when large lumps settle, they cause turbulence in the surrounding solution which, in turn, affects the readings. The fact that only a few eggs remain single adds to the difficulty. For such reasons, it seems to be better to determine the iso-electric concentration of cerium solution from the smoothed curve such as is shown in Figure 1.

TABLE 3

*The zeta potentials of the unfertilized eggs of Strongylocentrotus pulcherrimus (without jelly) in various mixtures of 1/2 M NaCl and 1/4 M CeCl<sub>3</sub>, the ratios of the mixtures being expressed in Ce concentrations in the resulting mixtures. The values are given in millivolts with standard errors.*

1/2 M NaCl	-39.7±0.67
NaCl + 1/3000 M CeCl <sub>3</sub>	-22.7±0.58
NaCl + 1/1000 M CeCl <sub>3</sub>	-13.3±0.57
NaCl + 1/100 M CeCl <sub>3</sub>	- 0.4±0.80
NaCl + 1/80 M CeCl <sub>3</sub>	+17.2±0.49
NaCl + 1/50 M CeCl <sub>3</sub>	+20.3±0.54
NaCl + 1/10 M CeCl <sub>3</sub>	+32.3±0.88
1/4 M CeCl <sub>3</sub>	+41.8±0.80

## DISCUSSION

The result reported in the first section of this paper led the author to the conclusion that, contrary to the case of *Arbacia*, the surface of the *Strongylocentrotus* eggs remains inert irrespective of whether calcium ions are present or absent in the surrounding medium. However, this statement signifies at the same time that the change in the zeta potential observed must be brought about by the adsorption of the calcium or cerium ions. In fact, the courses of both calcium- and cerium-curves are quite like any adsorption isotherms, but this situation can be analyzed further.

It is generally understood that the effects of ions on the zeta potential are two-fold. One is the effect of ions on the thickness of the diffuse layer (in Gouy's sense) and the other is the adsorption of ions to the surface (Müller, 1933). Fortunately, in such concentrated media as used here, the first proposition is practically negligible and it is permissible to consider solely the second proposition. The next point is that the solutions studied here are not solutions of a single salt but mixtures of two salts. In the Na-Ca series, as the calcium concentration is raised, successively three ions of sodium are replaced by two ions of calcium, and in the Na-Ce series, two ions of sodium are exchanged for one ion of cerium. Besides this, in the cerium series, the hydrogen ion concentration is also changed. But Rona and Michaelis (1919), after studying the adsorption of binary mixtures of salts by charcoal, came to the conclusion that when two salts are present, the more adsorbable ions drive the less adsorbable ions out of the surface so that the net result is very much like the adsorption of a single salt. This simplified picture may be applicable for the present case, since the adsorbability of trivalent cations far exceeds that of monovalent ions for a negatively charged surface.

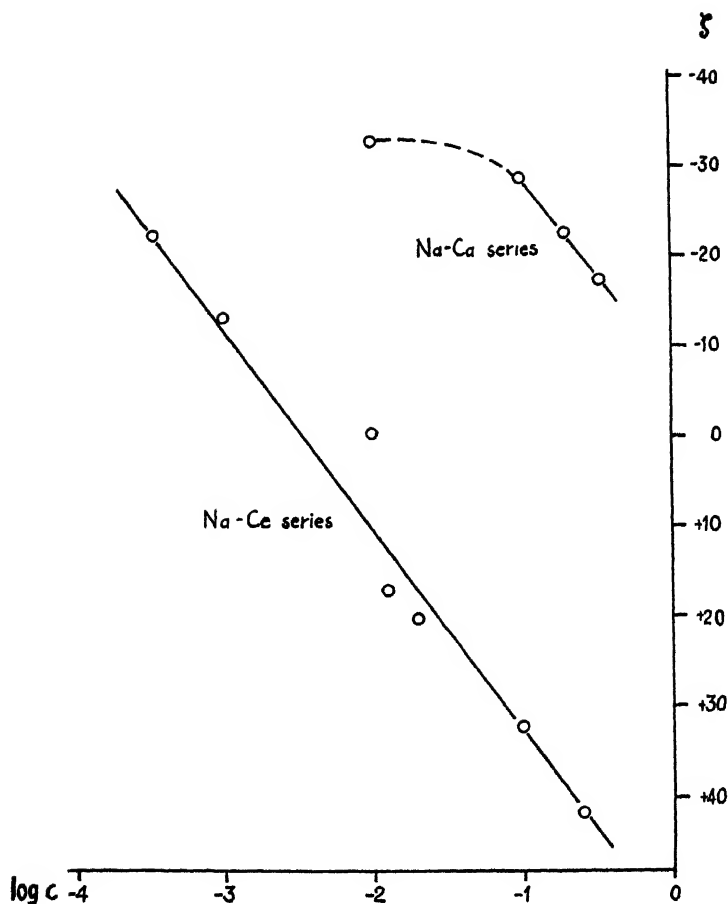


FIGURE 2. The plotting of the potential values against the logarithm of the concentrations. The Ce-series makes a straight line. The Ca-series does so only in the high Ca range.

Allowing this much simplification, the data can be checked by a formula for adsorption. Freundlich's adsorption isotherm will be used for testing, which is

$$a_{\pm} = \alpha \cdot c^{1/n},$$

where  $a$  is the quantity adsorbed,  $c$ , the concentration,  $\alpha$  and  $n$  are constants (see Freundlich, 1930, p. 244). As long as this formula holds, plotting of  $\log a$  against  $\log c$  ought to give a straight line. For electrokinetics, instead of  $a$ , the charge density  $\sigma$  must be used. But since  $\sigma$  is a function of  $e\zeta$ , in place of  $\log \sigma$ , the potential value  $\zeta$  can be directly plotted (see also Müller, 1933).\*

\*  $\sigma = \pm \sqrt{\frac{DKI}{2\pi} \sum n^0 (e^{-2e\zeta/KT} - 1)}$ , where  $\sigma$  is the charge per  $\text{cm}^2$ ;  $D$ , the dielectric constant;

$K$ , the Boltzmann's constant, namely,  $R/N = 1.37 \times 10^{-16}$ ;  $T$ , the absolute temperature;  $n^0$ , the number per  $\text{cm}^3$  of ions of valency  $Z$  far removed from the surface. In other words,  $n^0$  is connected to the molar concentration  $c$  with the relation of  $n^0 = c \cdot 606 \times 10^{-20}$ .  $e$  is the base of natural logarithms;  $e$ , the electronic charge.

The curve thus plotted is shown in Figure 2. As is evident, the cerium series makes a straight line (neglecting the value in  $\frac{1}{100}$  M). This can be taken as a proof that the change in potential in cerium solutions is due to the adsorption of the cerium cations. A similar plotting for the calcium series is also shown in Figure 2. In this case, although a straight line is obtained for the higher calcium region, it deviates in the lower concentrations. This can probably be interpreted as indicating that in the low calcium region, the effect of sodium ions is no longer negligible because the difference in adsorbability and in the potency in influencing the zeta potential between sodium and calcium is not so great as between sodium and cerium.

The next point of importance is the agglutination of the eggs under the isoelectric condition. However, since this problem will be taken up separately in a following paper, the discussion will be deferred until then.

#### SUMMARY

1. The electrokinetic potentials of the unfertilized eggs of *Strongylocentrotus pulcherrimus* were measured in mixtures of various ratios of isotonic NaCl and  $\text{CaCl}_2$  and of NaCl and  $\text{CeCl}_3$ .
2. In the Na-Ca series, as the concentration of Ca-ions in the mixture was raised, the absolute magnitude of the negative potential of the eggs decreased. In the Na-Ce series, with the increase in concentration of Ce-ions, the sign of the zeta potential was reversed.
3. At the isoelectric point, the eggs agglutinated.
4. The course of the potential change can be attributed to the adsorption of the cations.
5. The existence of a covering layer and its dissolution in the absence of Ca-ions such as were seen in *Arbacia* eggs have not been found in *Strongylocentrotus* eggs.

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# ELECTROKINETIC STUDIES OF MARINE OVA. VII. RELATION BETWEEN THE ZETA POTENTIAL AND ADHESIVENESS OF THE CELL MEMBRANE OF SEA-URCHIN EGGS

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In the field of colloid chemistry, the electrokinetic potential is a factor of paramount importance for the stabilization of suspensions and emulsions. In a suspension of a single substance the stability is minimum in the isoelectric condition.

Agglutination of living cells on abolishing the surface potential has been observed by many investigators in blood cells (Coulter, 1920, 1922; Northrop and Freund, 1924; Oliver and Barnard, 1925) and bacteria (Northrop and De Kruif, 1922a, 1922b; Eggerth, 1923; Shibley, 1924; Falk, 1928; Mudd, Nugent and Bullcock, 1932; Mudd, 1933), and also in some plant cells (Pfeiffer, 1933, 1934). The object of the present paper is to see whether a similar relationship exists between potential and agglutination in such large cells as sea-urchin eggs and to investigate it from the standpoint of surface adhesiveness rather than of agglutination. In the majority of agglutination studies, it is conventional to give the critical potentials for agglutination. The critical potential however fails to give us adequate information about the physical state of the surface in the pre-agglutination states. The author thinks, for this reason, that the study of adhesiveness is better since it leads to a wider viewpoint. Studies of this kind are surprisingly few in the literature, presumably because of the inadequacy of methods of measuring adhesiveness (see Pfeiffer, 1935).

## METHOD

In the experiments to be reported, the stream method was adopted. The simple glass apparatus which was constructed is diagrammatically shown in Figure 1. It consists of a series of tubes, each square in cross section but differing in dimension. The end with the largest bore is connected to a large flat reservoir (omitted from the figure). This end serves as the inlet, and at the opposite end, there is an outlet tube provided with a stop-cock. As is evident, for a single run of this outfit, five different fluid current strengths can be obtained, the current strengths being calculated by the amount of liquid flowing through the tube during a definite time interval. By combining a choice of different bores and different pressures, it is easy to get a sufficiently wide range of current strengths. Then by taking the percentages of remaining cells over the initial numbers for different current strengths, a curve can be drawn. This curve will be referred to as the "remainder-curve" in later paragraphs. The area circumscribed by the remainder curve and the two axes indicates the magnitude of the surface adhesiveness characteristic to the conditions under investigation.

The actual procedure of the experiment was as follows: a suspension of eggs of a suitable concentration was prepared, introduced into the inverted apparatus, and the eggs were allowed to settle and lie undisturbed for 10 minutes. (The interval of 10 minutes was arbitrarily chosen but this interval was kept constant through all the experiments.) At the end of 10 minutes, the apparatus was reinverted (returned to the normal position), so that the eggs were now hanging from the ceiling, so to speak. Definite areas were then marked out along the midline of the tubes of each dimension, quite close to the outlet end, the total numbers of the cells included within the marked areas were counted, and the solution was allowed to flow for exactly 1 minute (interval arbitrarily chosen). The percentages of cells remaining after 1 minute's flow over the initial numbers in different current strengths give a remainder-curve. The positions of the marked-out areas were selected near the outlet end of each portion to avoid vortices in the flow near the inflow end caused by the narrowing of the tube. The distance from the inflow end necessary for obtaining a laminar flow is a function of the dimension of the tube and the current strength and can be calculated by the Reynolds number often used in hydronics.

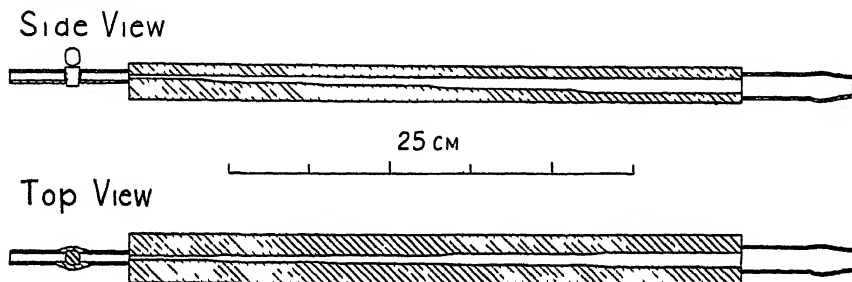


FIGURE 1. A diagram of the apparatus for measuring adhesiveness of the cell membrane. A series of tubes, square in cross section but differing in dimensions, is made through a glass bar (shaded). The large cylindrical tube on the right side is connected to a reservoir by rubber tubing. The small tube on the left side is an outlet and the current can be put on or shut off by the manipulation of the stop-cock. The eggs are made to adhere to the top wall in order to avoid the bumping of the washed-off cells into neighboring cells. It was desired to make the tube of the smallest dimension much longer, but this could not be realized because of a technical obstacle.

#### CRITICAL CONSIDERATION OF THE METHOD

*Current strengths.* In the data to be presented, the current strengths are calculated from the cross-sectional areas of the tubes and the amount of solution which flowed out during 1 minute. But, as is known, the current speed is not uniform throughout a cross-section, being maximal in the center and decreasing toward the walls. It is possible to treat this condition mathematically, but considering the other ambiguous terms involved such as 10 minutes' resting or 1 minute's streaming, the calculation was not attempted. Only the precaution was taken of selecting the positions of the marked-out areas along the midline of the top wall.

*Turbulences.* As was already mentioned, the object of marking out a definite area near the outlet end was to avoid the turbulences in the flow resulting from the narrowing of the tube. But this procedure could not eliminate the secondary turbu-



lences caused by the eggs themselves. In order to remove the errors of this source, care had to be taken to leave a certain distance between two adjacent eggs which could also be calculated. The distance necessary is the greater, the faster the current. As a matter of fact, if an adhering cell is observed by a microscope in a very strong current, the cell is sometimes seen to be vibrating, which will not happen unless it is being washed in a turbulent flow. Although no special attempt was made to remove errors of this kind, they were automatically minimized because of the fact that, in filling the apparatus with an egg suspension, the number of eggs which could get into a smaller tube, where the current speed was greater, was much less than that in a larger tube where the rate of flow was relatively slow.

*Glass surface.* The inner surfaces of the glass apparatus were polished in order to obtain as uniform a condition as possible and they were frequently cleaned with cleaning solution. But it soon came to the author's notice that the first reading after a cleaning was always higher than the succeeding readings. The readings from the second time on checked very well among each other. This is obviously due to the coating of the glass surfaces with proteins during the first measurement. For this reason, before starting a series of measurements, filling of the apparatus once with an egg suspension was practiced. Between successive measurements, the apparatus was washed with isotonic NaCl solution.

*Limit of the method.* The maximum current velocity tried in the present investigation was about 2m./sec. When the egg surface was made extremely adhesive by 1/200 M  $\text{CeCl}_3$ , even this flow speed could not wash off any cells. If microscopic observations were made of eggs in a current speed of around 2 m./sec., the eggs were seen to be deformed under the pressure exerted by the current. If the tearing force was further increased, finally the main bodies of the cells were torn off, leaving small portions of cytoplasm still adhering to the glass wall. This is not due to the failure of the membrane adhesiveness to resist the pressure but to the yielding of the membrane tension and the cohering force of the protoplasm to the force applied. This sets a limit to the method.

## RESULTS

*Hydrogen ion concentrations and adhesiveness.* During the work reported in a previous paper (Dan, 1947a), it was noted that when the concentration of hydrogen ions of the medium was increased, the sea-urchin eggs stuck together in various degrees roughly proportional to the concentration of hydrogen ions. In the literature, observations on the agglutination of sea-urchin eggs when an acid is added to sea water are frequently met with (Gray, 1916; Runnström, 1929; Vlès, 1924).

The present experiment was performed on the unfertilized eggs of *Anthocardius crassispina* from which the jelly had previously been removed. The pH values taken were 8.2 (natural sea water), 6.9, 4.7 and 2.7, salt corrections being made by Clark's table. The cataphoretic potentials at these pH's are -23.0, -26.3, -18.5 and -11.0 millivolts respectively (Dan, 1947a). The reasons for selecting these pH values are that the first is the natural medium, the second is the medium in which the absolute magnitude of the potential is the largest, the third corresponds to a point which is just above the critical potential for flocculation of the cells and the last is a very acidic medium. The most acidic medium investigated in the

previous work (pH 2.0) was intentionally avoided for a reason which will be discussed below.

One of the typical results of the adhesiveness measurements is shown in Table 1 A and B and Figures 2 A and B. It is to be noticed that although variation does exist in the adhesiveness of different batches, the general feature is very consistent.

TABLE 1  
*The remainder percentages in media of different pH's*

A	Stream method										Gravity method
Medium	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%
Sea water pH 8.2 (23.0 m.v.)	60.0	0.40	66.7	0.65	13.0	1.28	0	2.00	0*	8.00*	45.8
	49.1	0.40	37.5	0.65	16.7	1.28	3.8	2.00	0	8.00	67.0
Sea water pH 4.7 (-18.5 m.v.)	100.0*	0.41	100.0	0.68*	100.0*	1.33*	100.0*	2.08*	100.0*	8.33*	100.0
	100.0	0.58	100.0*	0.95*	100.0	1.87	100.0*	2.92*	100.0	11.53	100.0
	100.0*	3.74*	100.0*	6.19*	100.0	12.13	70.1	18.96	4.5	75.83	—
	100.0	3.89	100.0	6.43	83.8	12.60	45.3	19.69	0	78.75	100.0
	98.7	4.07	93.1	6.74	79.6	13.20	49.3	20.63	6.3	82.50	100.0
Sea water pH 2.7 (-11.0 m.v.)	—	—	100.0	6.80	98.6	13.33	93.7	20.83	81.5	83.33	100.0
	100.0	7.61	100.0	12.59	98.6	24.67	93.7	38.54	81.5	154.16	—
B	Stream method										Gravity method
Medium	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%
Sea water pH 8.2 (-23.0 m.v.)	94.7	0.35	88.3	0.58	67.1	1.13	44.3	1.79	9.5	7.08	7.6
	77.7	0.56	75.5	0.92	56.1	1.80	41.0	2.81	4.8	11.25	—
	100.0	0.33	98.1	0.54	84.9	1.07	73.3	1.67	3.7	6.67	4.9
Sea water pH 6.9 (-26.3 m.v.)	100.0*	0.33*	100.0*	0.55*	100.0*	1.08*	68.4	1.69	5.7	6.75	2.0
	100.0*	0.56*	100.0*	0.92*	100.0	1.80	65.3	2.81	2.9	11.25	—
	—	—	99.1*	0.54*	100.0*	1.07*	72.2	1.67	1.9	6.67	1.6
	—	—	99.1	0.95	98.1	1.86	54.6	2.91	1.9	11.67	—
Sea water pH 4.7 (-18.5 m.v.)	100.0*	0.37*	100.0*	0.61*	100.0*	1.20*	100.0*	1.87*	97.4	7.50	100.0
	100.0*	1.90*	100.0	3.13	100.0	6.13	100.0	9.59	57.9	38.33	—
	100.0*	2.06*	100.0*	3.40*	100.0	6.67	94.5	10.42	36.7	41.67	100.0
	100.0*	3.96*	100.0*	6.53*	100.0	12.79	87.3	20.01	3.3	80.01	—
Sea water pH 2.7 (-11.0 m.v.)	100.0	4.04	98.6	6.66	100.0	13.05	89.7	20.42	83.3	81.67	100.0
	—	—	97.2	11.22	100.0	21.98	89.7	34.39	73.3	147.51	—

\*, omitted from figure. {, one lot of eggs exposed to two different current strengths.

There are unmistakable differences between the adhesiveness in pH 2.7 and pH 4.7 and again between that in pH 4.7 and in the two higher pH values. But the difference in adhesiveness between the two higher pH values is hardly perceptible. This is caused by the fact that when the absolute magnitude of the potential increases well above the critical potential, the cells become so non-adherent that the present

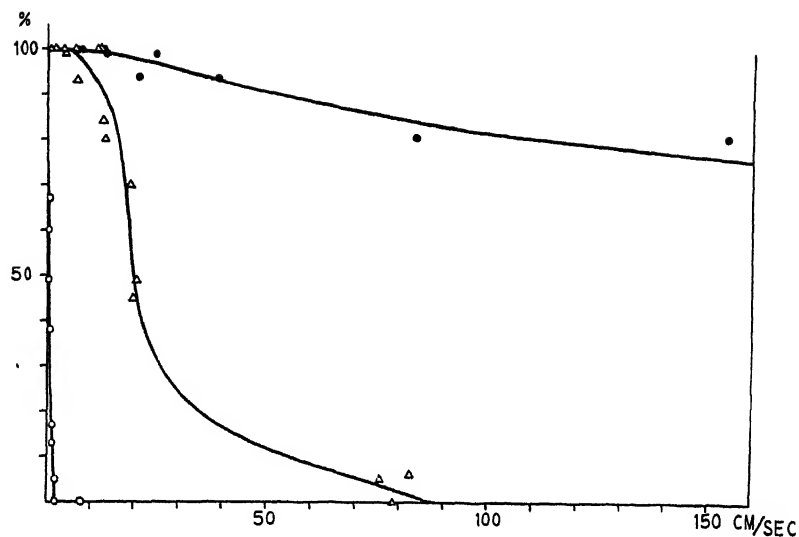


FIGURE 2A. The remainder-curves of *Anthocidaris* eggs in sea water of pH 8.2 (normal sea water) (○—○), pH 4.7 (△—△) and pH 2.7 (●—●). The ordinates are the percentages of the remaining cells, and the abscissae are the current strengths. The area circumscribed by a remainder-curve and the two axes is taken as the index of adhesiveness.

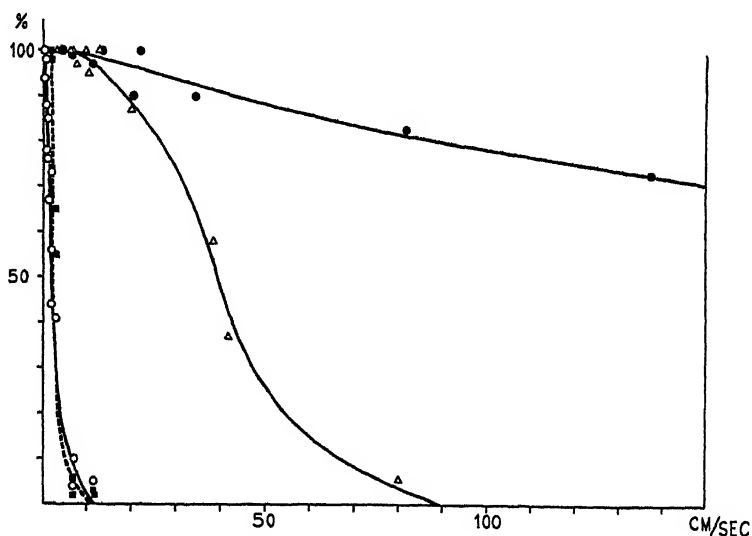


FIGURE 2B. Another set of *Anthocidaris* remainder-curves in sea water of pH 8.2 (○—○), pH 6.9 (■—■), pH 4.7 (△—△) and pH 2.7 (●—●), showing the reproducibility of the results among different batches. Note also that the adhesiveness in pH 8.2 and that in 6.9 are nearly the same although they can be well differentiated by the gravity method (see Table 1 B).

technique is too crude to show the difference. Fortunately, however, the difference can be caught by the gravity method. On inverting the container, the number of the cells which fall is greater for pH 6.9 than for pH 8.2 on many occasions (see Table 1 B). In pH 6, the inversion of the tube fails to tear off any eggs from the wall, which indicates that at pH 6, the adhesiveness suddenly increases. It is extremely interesting to remember here that the absolute magnitude of the surface potential in *Anthocidaris* eggs suddenly decreases between pH 7 and pH 6.

It was pointed out previously that the change in cataphoretic potential caused by the change in hydrogen ion concentration is always perfectly reversible. The change in adhesiveness is also reversible. An example is given in Table 2 and Figure 3. The curves for before and after the treatment with the medium of pH 4.7 overlap perfectly. One batch of eggs was met with which became unusually adhesive in the medium of pH 4.7, but even in that case, the reversibility was perfect.

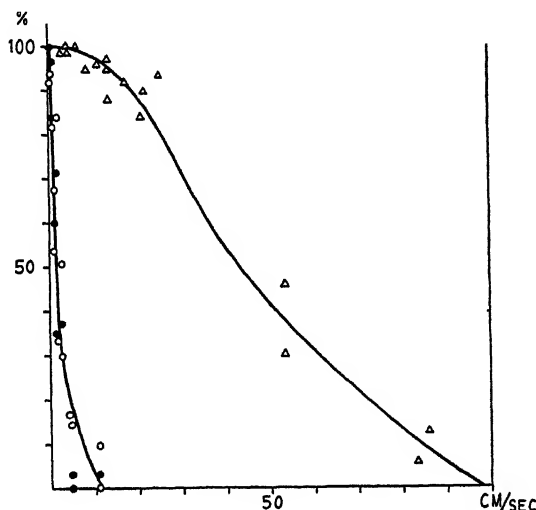


FIGURE 3. A set of records showing the perfect reversibility in the adhesiveness of the cell membrane of *Anthocidaris* eggs. ○ — ○, the remainder-curve in sea water of pH 8.2. Δ — Δ, that in sea water of pH 4.7. ● — ●, that in sea water of pH 8.2 after the treatment with acidified sea water of pH 4.7.

**Cerium ions and adhesiveness.** The results of the cataphoretic measurements of a Na-Ce series were taken from the preceding paper (Dan, 1947b). These cataphoretic measurements were not made under exactly the same conditions as the present adhesiveness measurements, but they are believed to be comparable.

An example of the adhesiveness measurements in a Na-Ce series is given in Table 3 and Figure 4. This figure is self-explanatory. When the cerium concentration becomes higher, i.e., as the absolute magnitude of the negative potential decreases, the remainder curves become higher and higher and after passing 1/200 M  $\text{CeCl}_3$ , which is nearly the isoelectric concentration, the curves come down again. A comparison of the cataphoretic values with the adhesiveness values is given in

TABLE 2

Medium	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.
Sea water pH 8.2 (-23.0 m.v.)	94.0*	0.08*	91.9	0.14	98.8*	0.27*	81.7*	0.42*	33.3	1.67
	94.0*	0.24*	90.0*	0.39*	95.0*	0.77*	67.5	1.20	14.3	4.79
	94.0	0.55	81.8	0.90	84.0	1.77	50.8	2.76	9.5	11.04
	100.0*	0.21*	100.0*	0.34	84.3*	0.67*	53.7	1.04	16.7	4.17
	100.0*	0.53*	96.2*	0.88	78.1*	1.73*	29.9	2.71	0	10.83
Sea water pH 4.7 (-18.5 m.v.)	100.0*	0.55*	100.0*	0.90*	100.0*	1.77*	98.5	2.76	95.8	11.04
	100.0*	0.85*	100.0*	1.41*	98.3*	2.77*	98.5	4.32	91.7	17.29
	100.0*	2.63*	93.2*	4.35*	96.6*	8.53*	97.1	13.33	45.8	53.33
	100.0*	4.24*	93.2*	7.01*	96.6*	13.73*	89.7	21.46	12.5	85.83
	100.0*	1.23*	100.0*	2.04*	100.0	4.00	100.0	6.25	93.3	25.00
	100.0*	2.63*	100.0*	4.36*	94.7	7.53	87.8	13.33	30.0	53.33
	91.7*	4.12*	96.3*	6.80*	94.7	13.33	83.8	20.83	6.7	83.33
Sea water pH 8.2 (-23.0 m.v.)	90.2*	0.24*	94.4*	0.39*	84.2	0.77	35.0	1.20	0	4.79
	100.0*	0.23*	100.0	0.37	82.1*	0.73*	60.0	1.15	3.1	4.58
	98.2*	0.55*	96.8	0.90	71.4	1.77	37.1	2.76	3.1	11.04

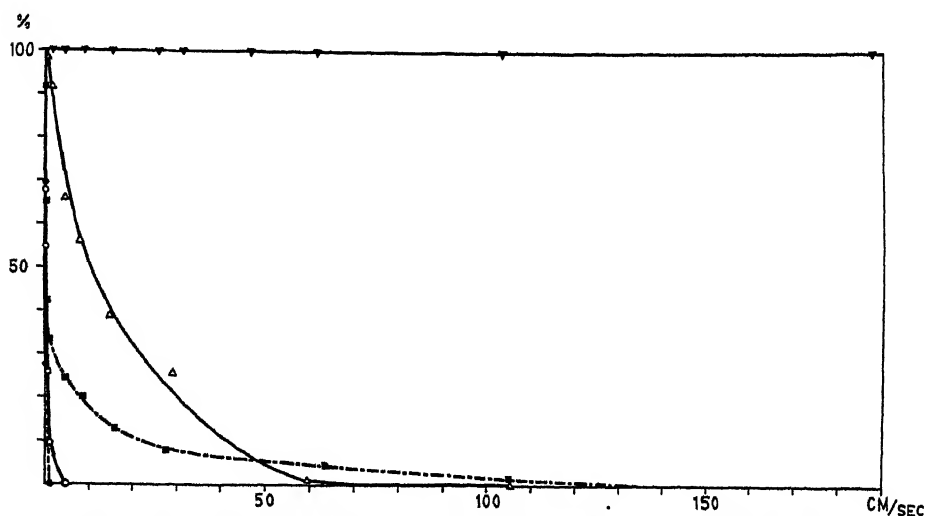


FIGURE 4. The remainder-curves of *Strongylocentrotus* eggs in 1/2 M NaCl (○—○), 1/3000 M CeCl<sub>3</sub> + NaCl (Δ—Δ), 1/200 M CeCl<sub>3</sub> + NaCl (▼—▼), 1/50 M CeCl<sub>3</sub> + NaCl (■—■) and in 1/10 M CeCl<sub>3</sub> + NaCl (●—●).

TABLE 3

Medium	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.
1/2 M NaCl (-39.7 m.v.)	67.7	0.24	54.7	0.39	5.7	0.77	9.4	1.20	0	4.79
1/3000 M CeCl <sub>3</sub> in NaCl (-22.8 m.v.)	97.7	0.57	91.2	1.86	66.0	4.61	56.0	8.13	38.9	15.06
	25.5	29.19	0.9	59.55	0	105.42				
1/200 M CeCl <sub>3</sub> in NaCl (+4.0 m.v.)	100.0	1.79	100.0	4.56	100.0	9.11	100.0	15.57	100.0	25.89
	100.0	31.53	100.0	46.88	100.0	61.67	100.0	103.54	100.0	187.50
1/50 M CeCl <sub>3</sub> in NaCl (+21.4 m.v.)	91.7	0.24	65.4	0.40	42.2	0.76	33.3	1.22	24.4	4.69
	19.9	8.69	12.8	15.91	8.0	27.71	4.5	63.75	1.7	105.00
1/10 M CeCl <sub>3</sub> in NaCl (+33.9 m.v.)	69.5	0.23	28.0	0.38	9.2	0.74	0	1.15	0	4.61

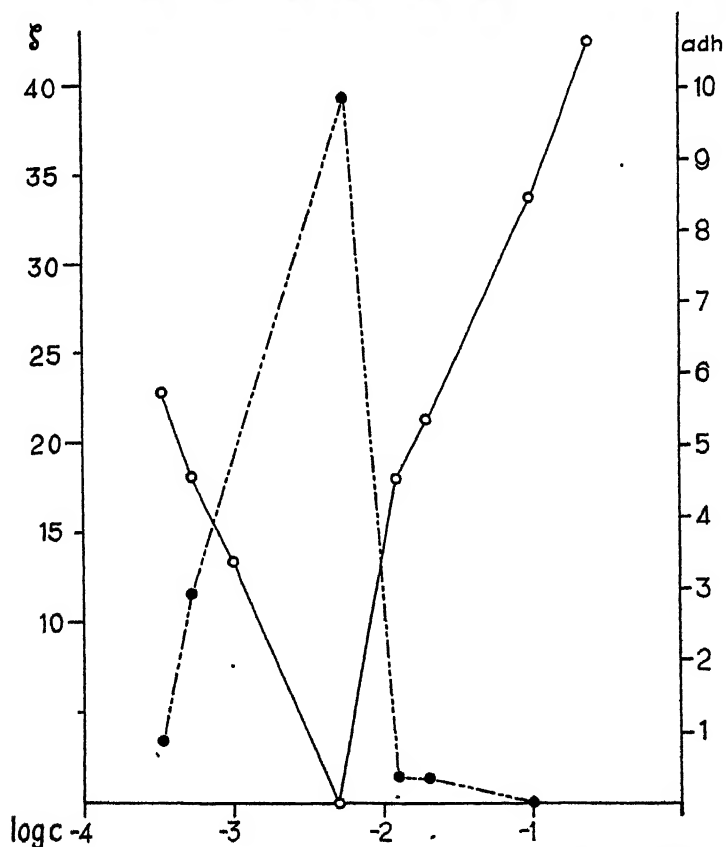


FIGURE 5. Inverse relation between the absolute magnitude of the zeta potential (○—○) and the adhesiveness (●—...—●) as plotted against  $\log c$  of cerium ions.

Table 4 and is represented graphically in Figure 5. Inverse correlation between the two properties is unmistakable. In this case, considering the crudeness of the technique, the adhesiveness values were roughly obtained by cutting out the plotted areas and weighing them instead of measuring the areas by a planimeter. The ratios of the adhesiveness values are in the order given in Table 4: e.g., 1:23.5:80.5: >>> 273.6:10.2; 9.3:0.7.

TABLE 4

Conc. of Ce-ions in mol.	O(NaCl)	1/3000	1/2000	1/1000	1/200	1/80	1/50	1/10	1/4
Zeta potentials in m.v.	-39.7	-22.8*	-18.1*	-13.4	+4.0*	+18.1	+21.4	+33.9	+42.6
Ratios of adhesiveness values	1.0	23.5	80.5	—	273.6	10.2	9.3	0.7	—

\* Potential values found by intrapolation.

#### SPECIFIC EFFECT OF DIFFERENT IONS ON ADHESIVENESS

If the cataphoretic potential is the only factor involved in determining the adhesiveness of the cell membrane, the eggs should have the same adhesiveness at the same potential, no matter by which ions the change is brought about. In order to test this, 1/3 M  $\text{CaCl}_2$ , 1/2000 M and 1/80 M  $\text{CeCl}_3$  in NaCl were selected and the adhesiveness in these solutions was tested. In all three solutions, the absolute magnitude of the potential is approximately 18 millivolts. The adhesiveness data are given in Table 5 and Figure 6. The figure shows that the above supposition is not true. Although the remainder curves in 1/3 M  $\text{CaCl}_2$  and 1/80 M  $\text{CeCl}_3$  follow a more or less similar course, that in 1/2000 M  $\text{CeCl}_3$  runs decidedly higher. Of

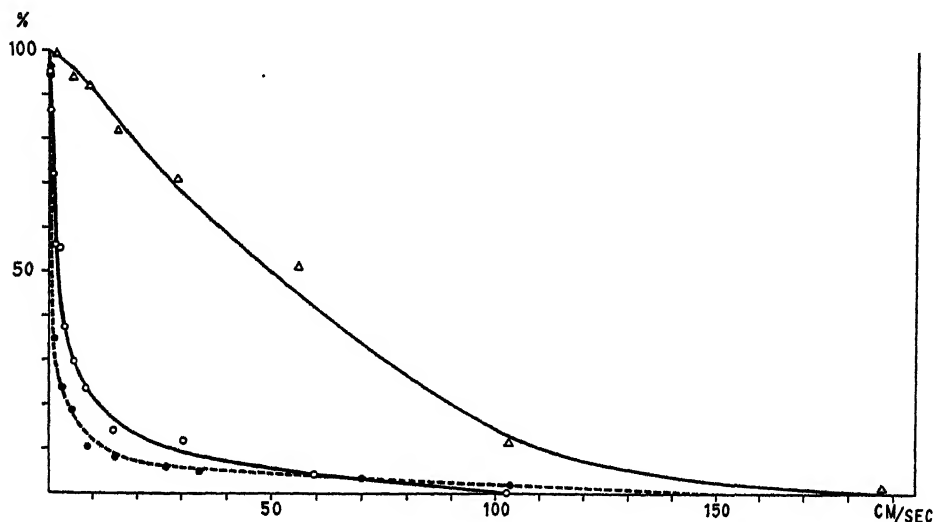


FIGURE 6. Comparison of the membrane adhesiveness of *Strongylocentrotus* eggs in three different media, in each of which the absolute magnitude of the cataphoretic potential of the egg surface is about 18 millivolts. ○—○, 1/3 M  $\text{CaCl}_2$ ; △—△, 1/2000 M  $\text{CeCl}_3$  + NaCl and ●.....●, 1/80 M  $\text{CeCl}_3$  + NaCl.

course, there is still room for the argument that since 1/2000 M cerium corresponds to the steepest part of the concentration-potential curve (see Dan, 1947b, Fig. 1), the greater adhesiveness here obtained may be due to a slight deviation of the cerium concentration to the more concentrated side. But a study of the acid series definitely negates this possibility. In a preliminary measurement, at pH 3 where the zeta potential of *Strongylocentrotus* eggs is -19.6 millivolts, the adhesiveness turns out to be much higher than that in 1/2000 M  $\text{CeCl}_3$ .

TABLE 5

Medium	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.	%	cm./sec.
1/3 M $\text{CaCl}_2$ (-17.5 m.v.)	95.1	0.26	86.5	0.44	71.9	0.81	55.9	1.33	55.4	2.39
	37.4	3.58	29.5	5.58	23.3	8.25	14.0	14.60	12.0	30.42
	4.1	59.37	0	102.50						
1/2000 M $\text{CaCl}_2$ (-18.1 m.v.)	99.3	1.74	93.9	5.40	91.9	9.20	81.8	15.58	70.9	29.22
	51.0	56.01	11.5	103.15	1.1	187.50				
1/80 M $\text{CeCl}_3$ (+17.2 m.v.)	94.3	0.25	96.3	0.41	68.5	0.77	34.6	1.28	23.3	2.92
	18.5	5.10	10.4	8.80	8.1	15.01	6.1	26.53	5.0	33.75
	3.3	70.21	1.8	103.33						

The above finding is not surprising considering the fact that Northrop and De Kruif (1922a) came across the same sort of phenomenon. They conclude that within 0.01 to 0.1 N of a salt solution, the potential is a primary factor for adhesiveness, but above 0.1 N, the specific effect of individual ions begins to modify the general issue.

Another complication to be remembered is a biological factor. Reports can be found in the literature to the effect that some cells normally have no detectable potential yet are not sticky at all (Mudd, 1933). Among marine ova, *Cerebratulus* eggs belong to this class (Dan, 1934). Besides this, adhesiveness measurements show that immature eggs with germinal vesicles are stickier than ripe ones, with no difference in their potentials. In bacteriology, this secondary modification of the potential-adhesiveness relation is attributed to the state of hydration of the surface. At present, no evidence is available whether or not such a situation exists among marine ova.

At any rate, it can be concluded that so far as sea-urchin eggs are concerned, the cataphoretic potential is a predominant factor in determining stickiness in the majority of the cases, and that only under certain circumstances can biological factors and ionic conditions modify the general trend to a lesser extent.

#### DISCUSSION

In the preceding paragraphs, evidences have been put forward to show that the adhesiveness of the cell membrane of sea-urchin eggs is primarily determined by the electrical condition of the membrane.

When the zeta potential is shifted, the adhesiveness also changes. Therefore, it is evident that referring only to the critical potentials of flocculation is not a satisfactory method. This shortcoming can be supplemented by the present technique.



However, for the sake of comparison, if the critical potential of *Strongylocentrotus* eggs is suggested, it must lie in the neighborhood of  $\pm 16$  millivolts, because in the cerium series,  $-13$  millivolts is decidedly below the critical point and  $-22$  and  $+21$  millivolts are above it, while in the acid series,  $-19$  millivolts is just above the critical point. This figure of 16 millivolts agrees very well with the other data obtained in a variety of materials.

From the standpoint of cell physiology, two points of particular interest will be commented upon. When the correlation between the abolition of the surface potential and the agglutination of cells was established beyond any dispute in bacteria and erythrocytes, it naturally led investigators to attempt to discover the same relation in leucocytes. However, the careful investigations of Fauré-Fremiet (1927a, 1927b, 1928) and Fenn (1922) revealed the fact that the situation is either very complicated or the correlation fails entirely to hold in the leucocytes of both vertebrates and invertebrates. This fact may be taken as very instructive. As is well known, bacteria and erythrocytes are comparatively inert cells, and as such, are predominantly fitted for the analysis of purely physico-chemical factors. Attempts for further advancements along this path might very likely have been attended with more success if they had been directed to egg cells rather than to leucocytes. The present work will offer a good example to bring out the contrast of the two materials.

The last question is how far the cataphoretic potential is connected with vital activity. In the present stage of our knowledge, no unanimity has been reached among investigators. Many of them even negate a possible correlation between the two. Yet, some positive evidences are accumulating. In the bacteriological field, it is often suggested that the virulence of pathogenic bacteria (Rosenow, 1933a, 1933b, 1934; Rosenow and Jensen, 1933) and the nitrogen fixing power of soil bacteria (Tittsler, Lisse and Ferguson, 1932) are correlated with the surface potential.

As for sea-urchin eggs, the data are too scanty to warrant a definite conclusion. But it is interesting to point out that Schechter (1937), in his study on the endurance of fertilizability of *Arbacia* eggs in mixtures of NaCl and CaCl<sub>2</sub> in various ratios, found that the eggs remained fertilizable longest in straight NaCl. This means at least in this case, that the longest viability is associated with the highest magnitude of the zeta potential. (In *Arbacia*, the dissolution of the calcium compound complicates the situation, but so far as the underlying surface is concerned, this statement holds. See Dan, 1936, Table II.)

This interdependence of high negativity and longevity of the eggs has an interesting connection with the electrokinetic conditions prevailing on the egg surface in a normal environment. In *Arbacia*, the potential on the internal granules is ca.  $-10$  millivolts, that of the protoplasmic surface is ca.  $-20$  millivolts, that of the calcium compound is ca.  $-30$  millivolts (Dan, 1936) and finally the potential of the jelly is ca.  $-35$  millivolts (Dan, 1933). In other words, the zeta potentials of the enveloping layers become higher in absolute magnitude in the order from the interior to the exterior. Among the data so far collected by the author, there is not a single exception to this arrangement (Dan, 1934). A priori thinking, where there is a barrier of a high zeta potential, more energy will be required for charged substances to pass through it, for substances carrying a charge the same in sign as that of the barrier will be repulsed at the boundary, while substances with the opposite sign will be tightly adsorbed. Thus preventing an unnecessary exchange of

substances with the environment, this condition may be effective in the protection of the cell interior. This is particularly significant for egg cells which have enough stored nutrient materials to live for a while independent of their surroundings. If we are allowed to go one step further in imagination, this fact may have something to do with the unexpectedly high values in the potentials of egg cells. But until further researches are made, this shielding effect by the high potential of the enveloping layer is simply offered as a suggestion.

### SUMMARY

1. A simple apparatus is described for measuring the adhesiveness of the cell membranes of sea-urchin eggs by the stream method.
2. By constructing a curve of the percentages of remaining cells in different current strengths (remainder curve), adhesiveness is expressed by the area circumscribed by the curve and the two axes.
3. In *Anthocidaris* eggs, when the zeta potential is changed by hydrogen ions, the adhesiveness changes correlatively and the shifts in the potential and the adhesiveness are both perfectly reversible.
4. In *Strongylocentrotus pulcherrimus*, both the potential and the adhesiveness change *pari passu* when cerium ions are added to the medium. Adhesiveness is maximum at the isoelectric point.
5. Specific effects of individual ions play a secondary role in determining the membrane adhesiveness.
6. Related phenomena are discussed.

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THE DEVELOPMENT OF THE SPIRACULAR CARTILAGES  
OF THE SPINY DOGFISH, ACANTHIAS  
VULGARIS (SQUALUS ACANTHIAS)\*

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The spiracular cartilages of Selachii differ in their number, shape and development among different forms. Concerning the morphological significance of these elements, there are two different views. The first is that of Huxley (1876) who considers the spiracular cartilage of *Heterodontus* (Cestracion) to be the separate otic process. Concerning this cartilage, he gives the following statement: "This small cartilaginous plate, which is connected only by ligament with the periotic cartilage above and with the quadrate below, answers to the otic process of the Frog's suspensorium."

Parker (1879) differentiates between the spiracular cartilages of sharks and skates by the following statement: "As a rule, the cartilage developed in the spiracular operculum in the shark is a ray (or rays); in the skate it is part of the body of the arch." Then he adds: "As to *Cestracion*, I quite agree with him (referring to Huxley) that the spiracular cartilage is the separate otic process, and it is worthy of notice that whilst the spiracular cartilages of the sharks are mere rays, yet they are chondrified detachments of the thin edge of the tissue that fills the primary fold in front of the first cleft."

So while he considers the spiracular cartilages of sharks to be mere rays, yet he agrees with Huxley in considering the spiracular cartilage of *Heterodontus*, which is also a shark, to be the separate otic process.

Edgeworth (1925) states that the spiracular cartilage of both *Scyllium* and *Heterodontus* is formed as a procartilaginous upgrowth of the hind end of the palatoquadrate which subsequently separates and chondrifies. From this he concludes that this cartilage is homologous with the otic process.

In dealing with the skull of *Torpedo*, De Beer (1926) describes a single spiracular cartilage which is connected with the posterior part of the palatoquadrate by dense tissue, and he is inclined to believe that it represents the otic process.

The otic process of the amphistylic *Notidanidae* is described by Goodrich (1909) to articulate with the post-orbital process of the auditory capsule and to pass outside and above the hyomandibular nerve.

The spiracular cartilage does not show these relations which are typical of an otic process. As a matter of fact, it lies far behind and much below the post-orbital process and it is separated from the latter structure by the large levator maxillae superioris muscle. The fact that it lies posterior and somewhat mesial to that muscle shows, as Allis (1914) points out, that it cannot be a part of the otic process.

\* Part of a thesis approved for the degree of Doctor of Philosophy in the University of London.

Against this view also is the fact that whereas there is a single spiracular cartilage in such forms as *Heterodontus* and *Scyllium* there may be two of these cartilages as in *Acanthias* or even three as in *Centrophorus*. The presence of two or more spiracular cartilages in some Selachians is by no means in favour of Huxley's interpretation.

The other view concerning the spiracular cartilages of Selachii is that of Gegenbaur (1872), who considers these cartilages to be modified mandibular rays.

In supporting this view, Holmgren and Stensio (1936) state that the spiracular cartilages must be interpreted as branchial rays of the mandibular arch. They think that their similarity to the branchial rays is perhaps most obvious in *Scymnus*, where they are represented by two flat, rod-like cartilaginous plates parallel to each other.

The development of the spiracular cartilages in the spiny dogfish, *Acanthias vulgaris*, favours Gegenbaur's view to a great extent and it shows thoroughly well that these structures are mandibular rays. They are developed in relation to the anterior spiracular wall to which they give support and they have always been found to have the same relation to the mandibular arch as the ordinary branchial rays to their corresponding arches.

They resemble the ordinary branchial rays, not only by their general appearance, both being slender, rod-like structures, but also by the fact that they chondrify at a later period than the mandibular arch. This is quite the same in the case of the branchial rays, the chondrification of which always takes place after that of their corresponding arches.

It has been observed that in a 39 mm. embryo of *Acanthias vulgaris*, where both the palatoquadrate and Meckel's cartilage are present as chondrified elements, the spiracular cartilages have not yet appeared as such. At this stage only the outer spiracular cartilage is present (Fig. 1A, *o.sp.c.*). It is in the form of an elongated mesenchymatous band which stands, more or less, vertically above the posterior end of the palatoquadrate. This band is well-defined and it stains deeper than the surrounding connective tissue from which it is, therefore, easily detectable. It is curved in such a way that its outer surface is convex and its inner one is concave. Its ventral end fits closely on the dorsal surface of the palatoquadrate, nearer to the outside than to the inside. Inner to this spiracular cartilage, is to be seen in the section, the spiracular pouch which is extending, more or less, obliquely and which is connected with the pharynx by an elongated, narrow tube. It opens to the outside in a more posterior section.

The spiracular cartilages were then studied in a 45 mm. embryo, the cartilaginous skeleton of which has been stained with methylene blue by Van Wijhe's method (1902) and micro-dissected. At this stage, not only the outer spiracular cartilage is present, but also the inner one. But they are not present as true cartilaginous structures since they have not become stained with the methylene blue. The latter stain, as Van Wijhe pointed out, differentiates not only between cartilage and other tissues, but also between cartilage and procartilage. Although the spiracular cartilages did not become stained with the methylene blue as other parts of the cranium in which chondrification proceeded, they are more rigid than the surrounding tissues and could be easily shown in the dissection.

They are present at this stage as two slender procartilaginous rods which form

nearly right angles with the posterior part of the palatoquadrate (Fig. 2, *i.sp.c.* and *o.sp.c.*). They are somewhat curved towards the posterior end of the animal and their ventral ends are in contact with the upper surface of the palatoquadrate.

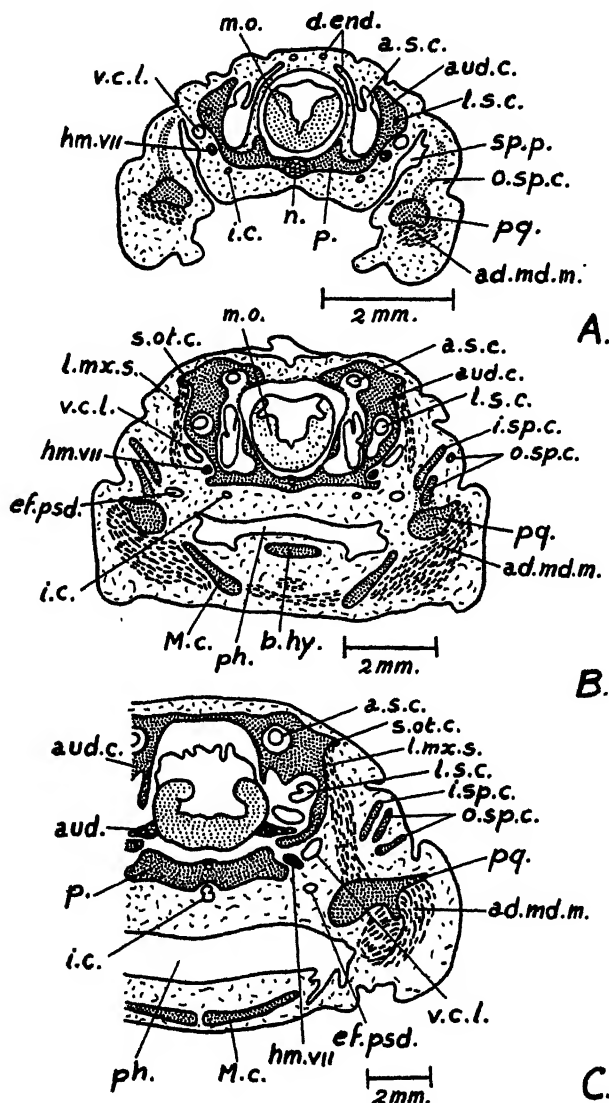


FIGURE 1. Transverse sections in the head region of *Acanthias vulgaris* showing the spiracular cartilages of: A, 39 mm.; B, 64 mm. and C, 110 mm. embryos.

These ventral ends are broader than the dorsal ones which are pointed. The outer spiracular cartilage, which, as stated above, appears earlier than the inner one is more elongated than the latter element. Their relation to the palatoquadrate, is

here strikingly similar to that of the hyal rays to the hyomandibular and ceratohyal, as well as to that of the branchial rays to both the epibranchials and ceratobranchials.

Mori (1924), in describing a 44 mm. embryo of *Acanthias*, states that there is a single spiracular cartilage which is oval in shape and which has its longer axis parallel to that of the palatoquadrate. This description is not correct as it has been found that the spiracular cartilages are always, in *Acanthias*, rod-like structures which stand at right angles to the palatoquadrate.

Although Sewertzoff (1899) describes also a single spiracular cartilage in his 50 mm. stage, yet his description of this single element is in accordance with that of the 45 mm. embryo given above. This cartilage is rod-like and is at right angles to the palatoquadrate.

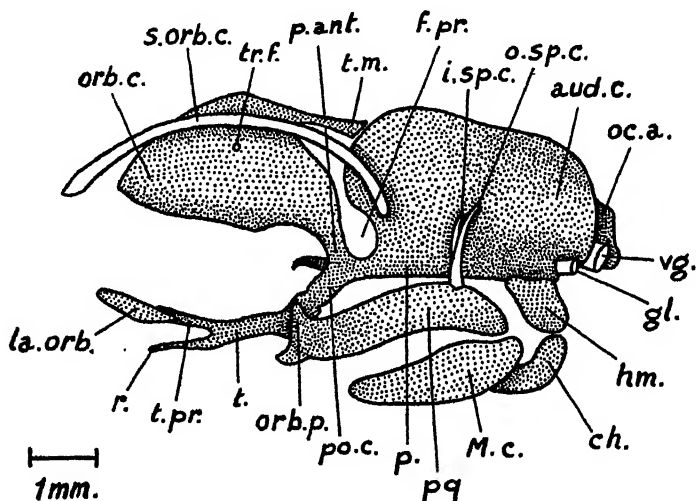


FIGURE 2. The chondrocranium of 45 mm. embryo of *Acanthias vulgaris* stained with methylene blue and micro-dissected to show the relation of the spiracular cartilages to the palatoquadrate.

In the following stage, which is a 64 mm. embryo, the two spiracular cartilages have chondrified and they still retain their approximation to the palatoquadrate (Fig. 1B). The outer spiracular cartilage is thicker than the inner and its base is closer to the palatoquadrate. The inner has grown more dorsally than in the previous stage. The ventral ends of both of them are surrounded by mesenchymatous tissue which extends ventrally to lie in contact with the dorsal surface of the palatoquadrate. Dorsal to the spiracular cartilages and lateral to the auditory capsule, is shown, in the section, the most posterior part of the levator maxillae superioris muscle (*l.mx.s.*). It is attached dorsally to the supra-otic crest of the auditory capsule which is, here, not yet well-developed.

In a later stage (102 mm. embryo) the development of the spiracular cartilages proceeded further by their growth in the dorsal direction, particularly the inner one which now exceeded the outer in length. Its dorsal end lies now opposite the upper surface of the lateral semicircular canal (Fig. 3A). The relation of the two spiracular cartilages to the palatoquadrate is the same as before and again the

outer lying closer to it than the inner. They are somewhat facing backwards and they lie right at the back of the levator maxillae superioris muscle.

Whereas these cartilages are quite simple rods in the previous stage, their structure is here rather interesting. The outer spiracular cartilage has a bifurcate dorsal end, the outer of the two branches is somewhat broadened to form a small lamella while the inner one is slender (Fig. 3A'). The dorsal end of the inner

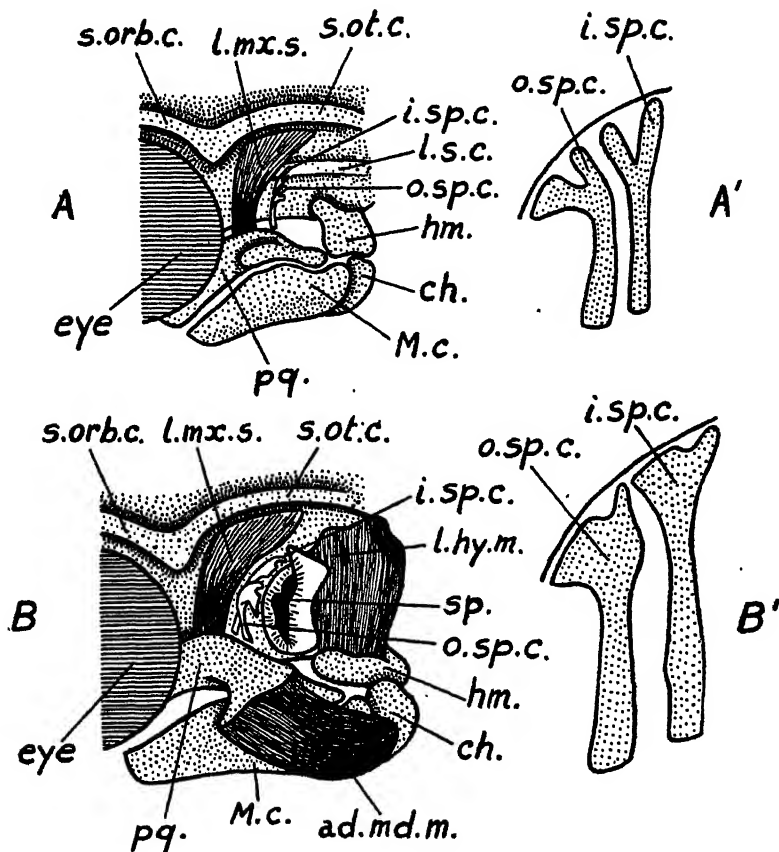


FIGURE 3. Lateral and posterior views of the spiracular cartilages of 102 mm. embryo of *Acanthias vulgaris* (A and A'); same of 270 mm. embryo (B and B').

spiracular cartilage is, on the other hand, divided into two branches which are nearly of the same thickness. The tips of the four branches lie, in the figure, on a curved line which represents the margin of the anterior spiracular wall.

The distinct bifurcation of the dorsal ends of the two spiracular cartilages is interesting owing to the fact that they show great resemblance to the branching hyal rays of the same stage. These latter rays are shown in Figure 4 where some of them are simple rods of cartilage while others have bifurcate posterior ends. Great similarity is to be observed on comparing the two spiracular cartilages with



these bifurcate hyal rays of the same individual. Each one of the latter structures possesses a simple stalk which lies opposite the ceratohyal and a bifurcate posterior end which lies away from it. The spiracular cartilages are strikingly similar to them and they differ only in extending dorsally instead of posteriorly as these rays extend. This similarity favours the view that they are nothing but mandibular rays formed to support the anterior spiracular wall.

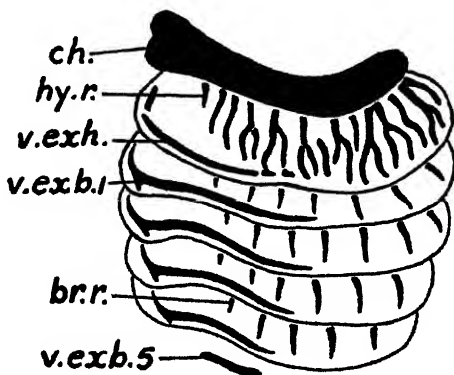


FIGURE 4. Hyal and branchial rays of 102 mm. embryo of *Acanthias vulguris* stained with methylene blue and micro-dissected.

The spiracular cartilages are also shown to be bifurcate in a still older stage (110 mm. embryo) which has been sectioned. In this latter embryo they are more developed than in the previous stage and they spread over a large area in the spiracular wall. The two branches of the outer spiracular cartilage are shown in the section (Fig. 1C, *o.sp.c.*). Of the inner one, only the outer branch is found in the same section, while the inner branch is to be seen in another section. Inner to them, there is the posterior part of the levator maxillae superioris muscle which extends from the supra-otic crest above to have its ventral insertion on the dorsal surface of the palatoquadrate. The latter cartilage is here well-developed and possesses a prominent ventral process which adds to the efficiency of the attachment of the adductor mandibularis muscle to that cartilage. Inner to the palatoquadrate there is the efferent pseudobranchial artery (*cf. psd.*) which extends from the spiracular pouch to unite with the internal carotid artery (*i.c.*) after piercing its way through the lateral cranial wall.

The bases of the two spiracular cartilages are present in a more posterior section where they lie quite close to the palatoquadrate cartilage, the same as in the previous stages. This section is chosen mainly to show the two branches of the outer spiracular cartilage which are extremely distinct.

Finally the spiracular cartilages have been dissected in a 270 mm. embryo, a stage in which the yolk sac has completely disappeared and the embryo is ready to leave the body of the female fish and lead a free life. They are here broader than before in the transverse direction, so that in a posterior view (Fig. 3B') they appear as two small plates of cartilage instead of being rod-like as in the previous stages. At the same time the bifurcation of their dorsal ends is about to disappear com-

pletely. This is brought about by the growth of the two branches of each spiracular cartilage towards each other to fill up the space lying between them. This growth has advanced in this stage to such an extent that only the tips of the original branches are to be observed. As a result, the dorsal part of each spiracular cartilage is much broader than its ventral part and it is present as a somewhat broad, flat lamella carried by the original stalk of each cartilage. The original bifurcation, though nearly completely obscured, is still to be detected at the upper margin of that lamella.

In a lateral view, the spiracular cartilages are strongly curved backwards, this curvature following that of the spiracular pouch (Fig. 3B). Their ventral ends are also in contact with the upper surface of the palatoquadrate.

The spiracular cartilages of this stage are quite similar to those of adult *Acanthias* where they are described by Wells (1917) as two small, flat cartilage bars. The only difference is the complete disappearance of the original bifurcation of their dorsal ends in the case of the adult fish.

The development of the spiracular cartilages in *Acanthias vulgaris*, therefore, supports completely the view expressed by Gegenbaur that these cartilages are simply mandibular rays. Summarizing the facts upon which this conclusion is based, they are as follows:

1. They chondrify at a later period than the mandibular arch which is in complete accordance with the chondrification of the ordinary branchial rays with respect to their arches.
2. They are, particularly at early stages of development, quite simple rods of the same shape as the branchial rays and they articulate with the palatoquadrate in a way which is similar to the articulation of the latter rays with their corresponding arches.
3. The fact that at a certain stage of their development they have bifurcate dorsal ends and that this bifurcation makes them quite identical with the bifurcate hyal rays of the same stage is a good support to that view.
4. At no stage of development whatsoever, have they been found to be in continuity with the palatoquadrate, a fact which is against the idea that they represent the separate otic process.
5. The fact that they lie posterior to the levator maxillae superioris muscle and far away from the post-orbital process shows that they do not occupy the position of a typical otic process.

When we compare the function of the spiracular cartilages, which are supporting the anterior spiracular wall, with that of the branchial rays, a great similarity between both structures is to be observed. The branchial rays are developed in relation with the gill lamellae to which they give support. It is well-known that the spiracular pouch represents the remains of an originally functional gill pouch which existed between the mandibular and hyoid arches and which was comparable with any of the following gill pouches. Such a functional spiracular pouch is described by Watson (1937) in the Acanthodians. It existed between the mandibular and hyoid arches with the result that the latter arch was free and did not function as a suspensorial element. In these latter fishes, not only the palatoquadrate but also

Meckel's cartilage, carries a complete set of mandibular rays. They are developed to support a mandibular operculum which is present in these fishes.

The existence of a functional gill pouch between the mandibular and hyoid arches is, therefore, from the palaeontological point of view, beyond reasonable doubt. When such a functional gill pouch has been reduced into the spiracular pouch during the phylogenetic development of the Selachii, it follows that the rays related to this pouch should also be reduced in number. In the latter fishes, therefore, it is to be considered that the spiracular cartilages are the remains of a complete set of mandibular rays which existed in their ancestral forms and which used to support a functional gill pouch lying between the mandibular and hyoid arches. Even after this reduction in number took place, the function is still the same and the spiracular cartilages are present to support the walls of the reduced spiracular pouch.

#### ACKNOWLEDGMENT

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#### EXPLANATION OF LETTERING

*a.s.c.*, anterior semicircular canal;  
*ad.md.m.*, adductor mandibularis muscle;  
*aud.*, auditory nerve;  
*aud.c.*, auditory capsule;  
*b.hy.*, basihyal;  
*br.r.*, branchial rays;  
*ch.*, ceratohyal;  
*d.end.*, ductus endolymphaticus;  
*cf.psd.*, efferent pseudobranchial artery;  
*f.pr.*, foramen prooticum;  
*gl.*, glossopharyngeal nerve;  
*hm.*, hyomandibular cartilage;  
*hm.VII*, hyomandibular branch of facial nerve;  
*hy.r.*, hyal rays;  
*i.c.*, internal carotid artery;  
*i.sp.c.*, inner spiracular cartilage;  
*l.hy.m.*, levator hyomandibularis muscle;  
*l.mx.s.*, levator maxillae superioris muscle;  
*l.s.c.*, lateral semicircular canal;  
*la.orb.*, lamina orbitonasalis;  
*M.c.*, Meckel's cartilage;  
*m.o.*, medulla oblongata;  
*n.*, notochord;  
*o.sp.c.*, outer spiracular cartilage;  
*oc.a.*, occipital arch;

*orb.c.*, orbital cartilage;  
*orb.p.*, orbital process of palatoquadrate;  
*p.*, parachordal;  
*p.ant.*, pila antotica;  
*ph.*, pharynx;  
*po.c.*, polar cartilage;  
*pq.*, palatoquadrate;  
*r.*, rostrum;  
*s.orb.c.*, supra-orbital cartilage;  
*s.ot.c.*, supra-otic crest;  
*sp.*, spiracle;  
*sp.p.*, spiracular pouch;  
*t.*, trabecula;  
*t.m.*, taenia marginalis;  
*t.pr.*, lateral trabecular process;  
*tr.f.*, trochlear foramen;  
*v.c.l.*, vena capitis lateralis;  
*v.exb.1-5*, first to fifth ventral extrabranchials;  
*v.exh.*, ventral extrahyal;  
*vg.*, vagus nerve.

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